APPLICATION OF MOLECULAR ANALYSIS TO THE DETECTION AND STUDY OF MINIMAL RESIDUAL DISEASE IN HAEMATOLOGICAL NEOPLASMS

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ABSTRACT

Haematological malignancies arise from the transformation of cells at various stages of development during haematopoiesis. Current treatment using standard chemotherapy or high dose chemotherapy and stem cell rescue leads to cure in a proportion of patients. However residual tumour may persist after treatment and in some patients relapse may result from growth of the original tumour. Detection of minimal residual disease was restricted by the low sensitivities of the techniques available. Detection of minimal residual disease in remission would have clinical application in monitoring an individual patients response to therapy and provide an early indication of relapse. Enzymatic amplification of DNA by polymerase chain reaction (PCR) can be used to identify minimal tumour. The aims of this study are i) to screen tumour from patients with leukaemia and lymphoma and determine the incidence of tumour markers, t(14;18) translocation, T-cell receptor δ (TcRδ) chain and immunoglobulin heavy chain (IgH) gene rearrangements ii) to develop sensitive PCR based techniques using these tumour markers and iii) to analyse serial remission samples and peripheral blood stem cells (PBSC) for residual tumour.

Southern blot analysis showed that 55% of patients with pre-B acute lymphoblastic leukaemia (ALL) had TcR Vδ2-Dδ3 rearrangements and that 85% of patients with B-lineage

disease had IgH rearrangements. PCR analysis showed a TcRδ marker in 53% of pre B ALL and a CDRIII marker in 77% of B-lineage disorders therefore these patients were available for further study of minimal disease. Direct sequence analysis of PCR products from TcR Vδ2-Dδ3 and the third complementarity-determining region (CDRIII) of IgH demonstrated sufficient junctional diversity to permit unique clone specific probes of 20 nucleotides to be designed. Junctional diversity was generated by random N-nucleotide insertion, gene segment deletion and addition of other D segments. In serial titration experiments, clone specific probes could detect one tumour cell in 10,000 to 100,000 normal cells. Clone specific probes were able to detect residual tumour in blood and marrow obtained during clinical remission. Serial analyses of remission samples showed residual tumour may either persist for up to 2 years before the onset of relapse or patients may become PCR negative at varying times during treatment and these patients continued to be in remission at 2 years. PCR analysis of PBSC demonstrated tumour in all of those collected from patients with pre-B ALL and in one third of patients with lymphoma. Concurrent bone marrow samples from NHL patients showed a higher frequency of PCR positivity than in PBSC indicating a potential advantage for their use in autologous transplantation.

DECLARATION

I declare that this thesis was composed by myself and consists entirely of my own work unless specifically indicated in the acknowledgements.

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Finally, I dedicate this thesis to my parents, who always provided support and encouragement.

ABBREVIATIONS

A	ampere
ALL	acute lymphoblastic leukaemia
(d)ATP	(deoxy) adenosinetriphosphate
AUL	acute undifferentiated leukaemia
BM(T)	bone marrow (transplant)
bp	base pair
C	constant region
CD	cluster designation
CDR	complementarity determining region
Ci	Curie
CML	chronic myeloid leukaemia
cps	counts per second
CT	computed topography
(d)CTP	(deoxy) cytidinetriphosphate
D	diversity segment
DFS	disease free survival
dH_2O	distilled water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FACS	fluorescence activated cell sorting
FCS	foetal calf serum
FR	framework region
g	gram
G-CSF	granulocyte colony stimulating factor

GM-CSF	granulocyte/ macrophage colony stimulating factor
(d)GTP	(deoxy) guanosinetriphosphate
GVHD	graft verses host disease
GVL	graft verses leukaemia effect
HD	Hodgkin's disease
HLA	human leukocyte antigen
hr	hour
IgH	immunoglobulin heavy chain
Ig κ	immunoglobulin kappa light chain
Ig λ	immunoglobulin lambda light chain
IL	interleukin
IMDM	Iscove's modified Dulbecco's medium
J	joining segment
kb	kilobase
kD	kiloDalton
l	litre
LFS	leukaemia free survival
m	month
M	molar
mbr	major breakpoint cluster region
μCi	microCurie
mCi	milliCurie
mcr	minor breakpoint cluster
μg	microgram
mg	milligram
MHC	major histocompatibility complex
min	minute
μl	microlitre

ml	millilitre
μm	micrometre
μM	micromolar
mM	millimolar
mmol	millimole
μmole	micromole
MNC	mononuclear cells
mol	mole
MRD	minimal residual disease
mRNA	messenger ribonucleic acid
N	nucleotide
ng	nanogram
NHL	non-Hodgkin's lymphoma
nm	nanometre
nM	nanomolar
nmol	nanomole
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PB	peripheral blood
PBSC(H)	peripheral blood stem cell (harvest)
PCR	polymerase chain reaction
PFGE	pulse field gel electrophoresis
pg	picogram
pM	picomolar
pmol	picomole
RNA	ribonucleic acid
RNase	ribonuclease
RSS	recombination signal sequence

Rx	therapy
s	second
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
TAE	Tris acetate EDTA buffer
TBE	Tris borate EDTA buffer
TcR	T-cell receptor
TdT	terminal deoxynucleotidyl transferase
TE	tris hydrochloride/ EDTA solution
TEMED	N,N,N,N,'-Tetramethylethylenediamine
TMACl	tetramethyl ammonium chloride
Tris	tris(hydroxymethyl)aminomethane
(d)TTP	(deoxy) thymidinetriphosphate
U	unit
UV	ultraviolet
V(H)	variable segment (heavy chain gene)
V	volt

CHAPTER I

INTRODUCTION

1.1 Haematological malignancy

Haematological malignancies encompass a spectrum of diseases which affect various developmental stages within the haematopoietic compartment (Figure 1.1). The transformation of cells during haematopoiesis leads to a neoplastic proliferation of developmentally arrested clones. The development of cytotoxic chemotherapeutic agents in the treatment of disease has provided an improvement in prognosis and recent advances in the fields of immunology and molecular biology have enhanced our understanding of tumour biology. These disorders can broadly be divided into leukaemias which affect progenitor cells and lymphomas which have a more mature phenotype (Table 1.1).

1.1.1 Classification of leukaemia

Leukaemia results from the accumulation of myeloid or lymphoid precursors in the stroma of the bone marrow which then enter the peripheral circulation. Diagnosis is made on the presence of greater than 30% lymphoid blast cells in the bone marrow.

1.1.1.1 Acute lymphoblastic leukaemia

ALL results from the expansion of lymphoid progenitors

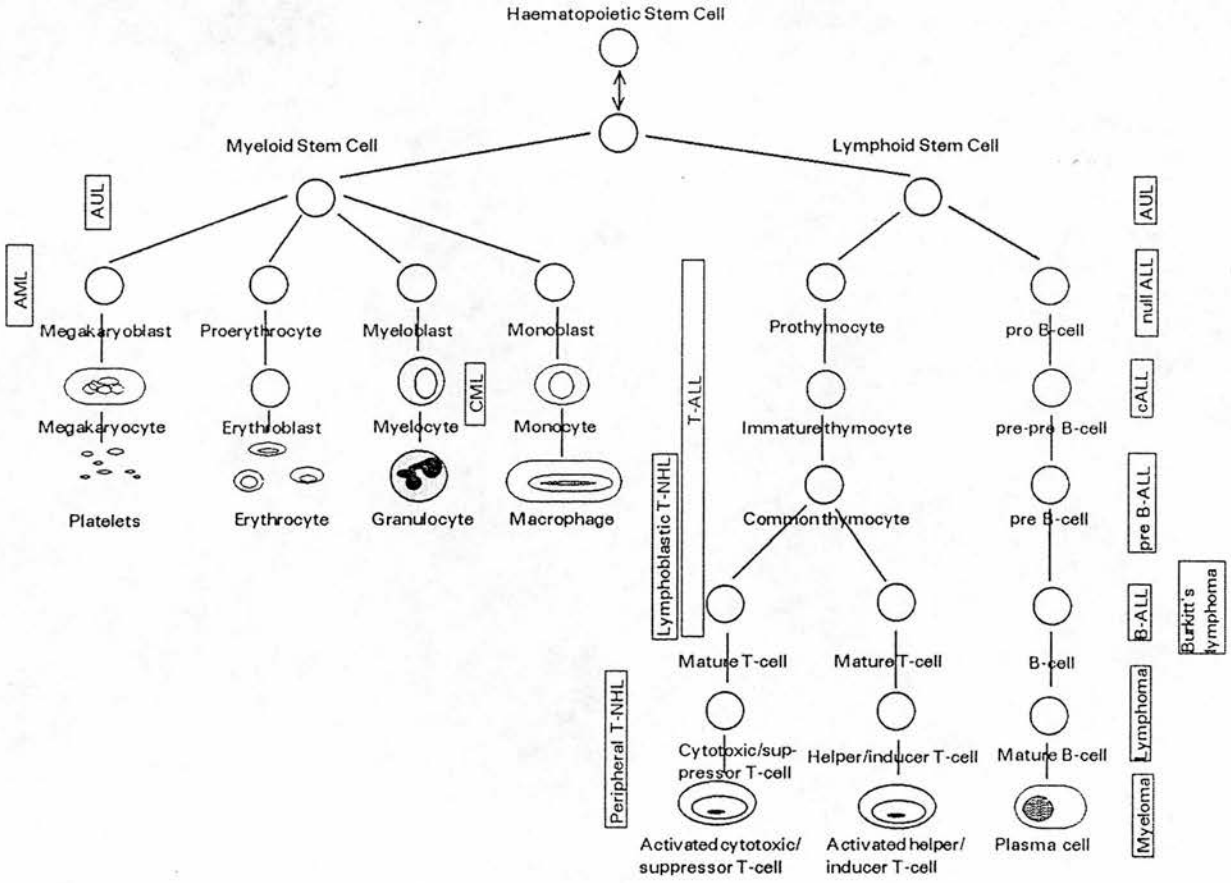


Figure 1.1 Differentiation pathway of haemopoietic cells. Disorders associated with particular compartments are indicated in vertical boxes (van Dongen, Adriaansen & Hooijkas 1988).

Disease	Incidence (per 100000 per year)	Age range (years)	Gene Rearr.	Chromosomal Abnormalities
B-lineage ALL	1	adult >15	IgH Igκ	t(4;11) t(9;22)
	4	childhood <15	Igλ TcRγ TcRδ	t(1;19) t(8;14)
T-ALL	1	all ages	TcRγ TcRδ	Tal-1
AML	1 to 4	increases with age	TcRδ IgH	t(15;17) t(9;22) t(8;21)
HD	2	1st peak 15-35 2nd peak >50	IgH TcRδ	
B-NHL	2.6-5.8 (T- & B-)	increases with age	IgH TcRδ	t(14;18) t(8;14)
T-NHL			TcRβ TcRγ TcRδ	14q11
Myeloma	7-20 (increases with age)	>50	IgH	

Table 1.1 Summary of age of onset and incidence of haematological malignancies (Harman 1991, Rosenthal 1991, Churchill 1991) . Associated gene rearrangements and the most common chromosomal translocations are also shown (Rubin and Rowley 1993, Greisser 1989, Felix and Poplack 1991).

and is a common disease of childhood (up to 16 years) accounting for 80% of acute leukaemias, though is less frequent in adults, seen in only 20%. Subtypes are classified by morphological and immunological criteria. B-lineage cell surface determinants and/or IgH chain rearrangements are found in 80% of cases, the remainder bear T-lineage markers. Expression of a coordinated series of cell surface determinants allows characterisation of the differential stage; the majority of B-lineage ALL express CD19 and CD10 and are classified as group III ALL (Foon and Todd 1986), whereas expression of CD19 without CD10 defines pro-B or null ALL. Group IV disease is associated with CD19, CD10 and CD20 expression, and together with group III constitutes common ALL. Cell surface immunoglobulin is found in mature B-cell ALL although this is relatively rare.

A range of cytogenetic abnormalities have been characterised which are of prognostic significance, including the t(9;22) Philadelphia chromosome which has been reported in up to 50% of adult and 5% of childhood cases (Maurer et al 1991); the t(4;11), frequent in ALL of infants (up to 1 year old) ; t(11;14) and t(1;9) (Rubin and Rowley 1993). Mature B-ALL is associated with the t(8;14) also found in Burkitt's lymphoma which results in the juxtaposition of the myc oncogene on chromosome 8 to the IgH locus on chromosome 14 (Shiramizu et al 1990). Although translocations usually indicate a

poor prognosis, polyploidy (greater than 50 chromosomes) is associated with a low risk group.

1.1.1.2 Acute myeloid leukaemia

AML results from the expansion of myeloid progenitor cells accounting for 80% of adult acute leukaemias though is less common in children. Differential stage is determined by morphological criteria although progressive expression of myeloid markers analogous to those in acute leukaemia can be detected by immunophenotypic analysis. Specific chromosomal abnormalities have been reported in particular subtypes, such as the t(15;17) found in most cases of acute promyelocytic leukaemia (Larson et al 1984).

1.1.2 Classification of lymphoma

Lymphomas are characterised by the proliferation of more mature lymphocytes which have migrated from the marrow to the lymph nodes, although neoplastic cells can be disseminated to both the marrow and the bloodstream. Subtypes are determined by morphological analysis of tumour biopsies.

1.1.2.1 Non-Hodgkin's lymphoma

NHL of both the T- and B-lymphoid compartments is found and can take an indolent (low grade) or aggressive (high grade) course depending on the maturity of the neoplastic cell (non-Hodgkin's lymphoma pathologic classification project 1982). Low grade NHL is characterised by a late

onset, whereas high grade disease is found in all age groups. Several chromosomal translocations have been described, the best conserved of which is the t(14;18) associated with up to 80% of low grade NHL and perhaps 5% of high grade disease (Weiss et al 1987). The t(8;14) found in mature B-ALL is also commonly associated with Burkitt's lymphoma, an aggressive high grade lymphoblastic disease.

1.1.2.2 Hodgkin's lymphoma

Demonstration of cells of a committed lineage associated with HD have not been consistently demonstrated although proliferations within involved lymph nodes are associated with an unusual binucleate cell, termed the Reed-Sternberg cell.

1.1.3 Myeloma

Myeloma originates from the deregulated division of activated B-lymphocytes, with a late onset increasing in incidence from the age of 50. A more mature form resulting in the expansion of terminal immunoglobulin secreting plasma cells in the peripheral blood is termed plasma cell leukaemia.

1.2 Treatment of haematological malignancies

Cytoreduction of tumour load provided by chemotherapy is dose dependent (Frei and Canellos 1980). Remission status is achieved if light-microscopy detects the presence of fewer than 5% leukaemic blasts (Bisel et al 1956), or if

no obvious evidence of disease is present on CT scan analysis in the case of lymphoma. Remissions are induced by chemotherapeutic agents possibly with radiotherapy to reduce bulk disease and are maintained by consolidation therapy. However a proportion of patients will go on to relapse due to the persistence of disease or resistance of the neoplastic cells to the drugs administered. Chemotherapy can induce remission in 90% of children with ALL and 60 - 80% of these will remain in remission for 5 years or more (Champlin and Gale 1989). Over 70% of adults enter remission of whom 20 - 35% continue in remission at 3 to 5 years. Similar figures are reported in AML (reviewed Craig 1990) with 76% of patients under 50 years of age achieving remission with a LFS of 25 to 40% at 3 years. Two thirds of patients with HD are cured by a combination of radiotherapy and chemotherapy, though 50% of NHL patients achieving remission eventually relapse and only 10% are cured. Few patients with myeloma achieve remission and those who do frequently relapse (Kyle, Greipp and Geitz 1986). Patients with refractory disease are candidates for transplantation.

Although the response of malignant cells to myeloablative therapy is dose dependent, the dose that can be administered is limited by toxicity to the bone marrow. In many cases sufficient therapy can be administered to provide long term remission or cure, however in those patients with refractory disease, bone marrow transplantation enables a very large dose of chemotherapy

to be given, followed by rescue of the bone marrow with donor haematopoietic stem cells. These stem cells can be derived from several sources, each with its own particular application.

Marrow collected from a sibling is used in allogeneic transplantation. The donor cells confer a graft versus leukaemia effect which can reduce tumour burden, as can the action of activated T- killer cells (Goldman et al 1988, Hamblin 1988). However a potentially lethal graft versus host reaction with varying degrees of severity is also observed.

An unrelated donor transplant is an option if an HLA matched sibling is not available (Ash et al 1990). Marrow is harvested from an HLA matched donor identified from a database of tissue typed volunteers, although this procedure is still in its infancy.

Another alternative is to use stem cells harvested from a patients own marrow whilst in remission, though this requires a relatively healthy and cellular marrow which has recovered from chemotherapy. Such autografts may still harbour neoplastic cells hence purging with monoclonal antibodies has been performed in an attempt to reduce this burden and minimise reinfusion of tumour which may promote relapse (Negrin et al 1991). The problems of hypocellular bone marrow and potential tumour contamination have been addressed with the development of

peripheral blood stem cell (PBSC) transplantation whereby mononuclear cells from the peripheral circulation are collected and stored (reviewed Craig, Turner and Parker 1992). PB does not normally contain significant numbers of pluripotent stem cells, but during regeneration following chemotherapy or in response to growth factors such as G-CSF, there is a large increase of these cells in circulation. Engraftment following transplantation is also more rapid, possibly due to the increased presence of mature leucocytes in the graft which reduce the period of depressed neutrophil counts during which time the patient is immunocompromised hence susceptible to infection.

Allogeneic transplantation can provide up to 50% LFS in ALL though this falls to 30% for autologous transplantation (Champlin and Gale 1989). A LFS at 5 years of 48% has been reported for AML patients undergoing allogeneic BMT compared to 36% at 7 years for autologous transplants (Craig 1990). Autologous transplantation in HD results in a 50% complete response in patients with disease refractory to chemotherapy . BMT in NHL shows survival at 3 years to be approximately 50%. Autologous transplantation with PBSC has been successfully used to reduce tumour load in myeloma and is better tolerated by older patients (Fermand et al 1989).

Although a greater reduction in tumour load is provided by transplantation, relapses still occur due to the

expansion of endogenous disease or from reinfused disease (in autologous transplantation) therefore the study of MRD in patients undergoing both chemotherapy and transplantation will enable the response of disease to be closely followed and perhaps provide an early indication of relapse. In these patients, treatment regimes may be ultimately tailored to their individual requirements.

1.3 Minimal residual disease

At presentation, a patient with ALL may have a tumour burden of 10^{12} neoplastic cells (van Bekkum 1984). After induction therapy, 90% of patients with ALL achieve remission, defined by the detection of less than 5% lymphoid blasts in a bone marrow sample analysed by light microscopy. However, this still represents a large tumour burden of up to 10^{10} neoplastic cells. This persisting tumour is termed MRD although its definition has been refined as increasingly more sensitive tumour detection techniques become available. Like morphological analysis of BM smears, the sensitivity of cytogenetic analysis is limited at the 5% level. Dual colour immunofluorescence analysis is applicable to a subgroup of patients with leukaemia and skilled investigators can optimally detect one neoplastic cell in a population of 100000 normal cells in specific instances (Campana et al 1990a), however changes in the expression of cell surface markers can lead to false negative results.

Likewise, patients with lymphoma or myeloma may have

persisting tumour cells in circulation when the patient has achieved clinical remission, the detection of which may have prognostic significance.

1.3.1 Molecular analysis

The application of recombinant DNA technology to the analysis of haematological malignancy has provided a great deal of insight into the biology of the disease as well as providing sensitive tools for the study of disease kinetics.

PCR facilitates the amplification of discrete regions of DNA many million times, hence is invaluable in the detection of very rare DNA or RNA species (Saiki et al 1985, 1988). The amplification of tumour specific translocations permits the sensitive detection of neoplastic cells, even when masked by large populations of normal cells.

The exploitation of immune receptor gene rearrangements and the diversity therein provide more widely applicable markers and enable the study of both T- and B-lymphoid disease, though are of less value in the analysis of myeloid disorders. The TcRδ locus is frequently rearranged on both T- and B-lineage leukaemia and has been applied to the detection of residual disease (Yokota et al 1991a), as has the TcR γ locus (MacIntyre et al 1990). Virtually all disorders of the B-lineage bear IgH rearrangements, hence this locus provides a widely

applicable marker for neoplastic cells (Yamada et al 1989, Trainor et al 1990).

1.4 Aims

I sought to investigate the use of Southern blot analysis with IgH and TcRδ chain genomic probes to characterise the frequency gene rearrangements in a group of patients with leukaemia, lymphoma and myeloma. These patients would then be screened by PCR analysis using primers specific for the t(14;18) in low grade NHL, IgH rearrangements in B-lineage disease and TCRδ chain rearrangements in both T- and B- lineage disorders. I then wished to refine these PCR systems to facilitate the sensitive analysis of disease persistence in patients undergoing either standard chemotherapy or transplantation. Tumour contamination in PBSC harvests would also be compared to that in autologous BM harvests to investigate if they provide a purer source of progenitor cells. Finally the prognostic value of PCR status would be evaluated.

CHAPTER 2

THE t(14;18) TRANSLOCATION

2.1 INTRODUCTION

2.1.1 Characterisation of the lesion

The t(14;18)(q32;q21) karyotypic abnormality is closely associated with low grade follicular NHL (Fukuhara et al 1978) though the reported incidence varies from 30 to 90% (Amakawa et al 1989, Weiss et al 1987). The translocation results in the juxtaposition of the bcl-2 oncogene on chromosome 18 next to one of the 6 IgHJ segments on chromosome 14 (Tsujimoto et al 1984). Although the neoplastic cell has a mature B-lymphocyte phenotype, the translocation is thought to occur at an early stage of pre-B cell development, possibly as the result of imprecise V-D-J recombination (Tsujimoto et al 1985). The signal sequences which would mediate this event have not been clearly demonstrated however, hence the precise mechanism has yet to be determined.

The majority of the breakpoints are found within a 2.8kb locus in the bcl-2 gene known as the major mbr which accounts for 30 to 70% of translocations studied (Pezzella et al 1990, Bakhshi et al 1987). This is located within the 3' untranslated region and does not

interrupt the coding sequence of the gene. A second group of breakpoints have been mapped to a site 20kb downstream of the mbr accounting for 25% of translocations and is known as the mcr (Cleary et al 1986).

2.1.2 Role Of Bcl-2

Transcription of the rearranged locus produces chimeric bcl-2/JH mRNA species which are translated into structurally normal bcl-2 protein molecules although the level of expression is elevated (Weiss et al 1987). Bcl-2 has been postulated to encode a low molecular weight G (GTP binding) protein involved in signal transduction (Haldar et al 1989) although homology with other G proteins such as Ras is weak (Reed et al 1988). The bcl-2 product has been localised on the inner surface of the mitochondrial membrane (Hockenberry et al 1990) and two species have been characterised, bcl-2α and 2β, 26 and 22kD respectively. They differ in their carboxyl termini due to differential splicing and have short membrane spanning domains but both lack leader or kinase domains (Reed et al 1988). Elevated levels of bcl-2 are normally found in early B-cell development but expression disappears with maturation (Tsujimoto et al 1989).

Mitogen induction was shown to increase bcl-2 mRNA levels in normal peripheral blood lymphocytes due to an increase in both transcription and stability of the message indicating the presence of both positive and negative regulatory elements in a complex mechanism (Reed et al

1987). Bcl-2 protein was shown to cooperate with c-myc in cellular transformation demonstrating oncogenic potential (Vaux et al 1988). As all t(14;18) lymphomas with rearranged bcl-2 show elevated transcription, gene transfer experiments to investigate the effect of overexpressed bcl-2 have been devised. Mice transfected with bcl-2/JH fusion genes develop a population of mature B-cells with a growth advantage (McDonnell et al 1989) but a murine fusion gene introduced into various factor-dependent cell lines will not confer long term survival in the absence of factor (Nunez et al 1990). High bcl-2 expression does, however, prolong short term survival of IL-3 and IL-4 as well as GMCSF dependent haemopoietic lines.

IL-3 deprived cells die via a highly regulated mechanism termed "apoptosis" hence bcl-2 may have a role in this programmed cell death pathway. This concept was supported by the finding that overexpressed bcl-2 resulted in the maintenance of an IL-3 dependent pro-lymphocytic B-cell line in G0 stage after IL-3 deprivation. Restoration of IL-3 allowed the cells to re-enter the cell cycle (Hockenberry et al 1990). Blockage of de novo protein synthesis with cyclohexamide also prevents death in the absence of growth factor supporting the existence of an active cell death mechanism. High levels of bcl-2 seem to interfere with the "programmed cell death pathway" prolonging lymphocyte survival which allows the accumulation of further genetic changes eventually

leading to neoplastic transformation.

A role was also demonstrated in maintenance of memory B-lymphocytes which maintain immune responses to specific antigens long after the initial exposure (Nunez et al 1991) and also postulated in Ig class switching (Amakawa et al 1991). The action of bcl-2 protein is not B-lymphocyte specific, immature thymocytes susceptible to apoptosis during positive selection in the thymus can be rescued by deregulated bcl-2 expression. Negative selection of self-recognising T-lymphocytes appears to be mediated by a bcl-2 independent pathway (Hockenberry et al 1990).

2.1.3 Southern Blot Analysis

The majority of low grade lymphomas with translocations can be detected by Southern blotting using a combination of mbr and mcr probes, with the advantage over cytogenetic analysis that viable tissue to provide metaphase spreads is not required (Zelenetz et al 1991). Southern blotting also allows other leukaemia or lymphoma subtypes to be assessed for t(14;18) translocations. Aisenberg et al (1988) reported that 19% of diffuse B-cell lymphomas had mbr bcl-2 rearrangements, whereas none could be detected in cases of small lymphocytic lymphoma or T-cell leukaemia or lymphoma suggesting that bcl-2 rearrangements are rare outwith NHL of follicle centre cell lineage. PFGE exploits electric field reversal and rare cutting restriction enzymes such as Not I to map

larger DNA fragments than are permitted by conventional electrophoresis. Even though the mbr and mcr of bcl-2 are 20kb apart they co-migrate on a 630kb DNA fragment, along with other breaks which could be even more distal, after Not I digestion (Zelenetz et al 1991). This means that a single digestion and single hybridisation with a bcl-2 probe will detect breaks in the bcl-2 gene more effectively than regular Southern analysis. The rare cases of low grade NHL negative by PFGE may indicate an alternative mechanism of bcl-2 activation or the existence of rare 5' breakpoints.

2.1.4 Polymerase chain reaction analysis

In addition to the methods described above, PCR of mbr and mcr loci can be performed to detect bcl-2/JH junctions. PCR will not detect as many translocations as PFGE but is of great value due to the vastly enhanced sensitivity it provides over all other techniques, which are limited by detection limits at the 1-10% level. Further to this archival material such as paraffin sections or stored BM slides can be assayed (Wright & Manos 1990, Fey et al 1987). The conserved nature of the majority of the translocation breakpoints provides an ideal locus for PCR analysis. Lee et al (1987) were the first to exploit the bcl-2/JH mbr junction sequence information and design bcl-2 and consensus JH amplimers. Using 12mer oligonucleotides, the Klenow fragment of DNA polymerase I and 30 cycles of amplification they reported a detection limit at the level of 1 tumour cell in 10^5

normal cells. Crescenzi et al (1988) used the thermostable Taq DNA polymerase to amplify the mbr region and demonstrated sensitivity at the level of 1 tumour cell in 10^6 normal cells after 45 cycles of amplification. Stetler-Stevenson et al (1988) used an mbr primer in conjunction with 3 JH primers; primer 1 was complementary to J1, 2, 4 and 5; primer 2 to J3 and primer 3 to J6. Sensitivity at the level of 1 in $2x10^5$ was reported. The mcr is also amenable to amplification (Ngan et al 1989) hence approximately 90% of patients with a t(14;18) can be assayed with these techniques.

2.1.5 Sequence Analysis

To enhance the specificity of the technique and investigate possible mechanisms mediating the rearrangement sequence analysis of the translocated region was performed. Sequencing of the mbr locus showed clustering breakpoints to within a few nucleotides. Random "N" nucleotide insertions characteristic of rearranged immune genes were also seen along with evidence for the occasional presence of D segments (Creszenczi et al 1988).

Eick et al (1990) sequenced mbr PCR products to generate patient specific amplimers derived from the unique N regions. They attempted to sequence the products either directly (using 2-5μg of DNA) or following a subcloning step. The former was more satisfactory as the high nucleotide misincorporation rate associated with Taq

polymerase was averaged out by sequencing the products of many reactions simultaneously. Asymmetric PCR which generates a single stranded product proved the most convenient as less target DNA was required per sequencing reaction.

To investigate the origin of N nucleotides observed at the translocation junctions, Cotter et al (1990) sequenced both the translocation and the reciprocal translocation junction. This was made possible by the observation that the 18q- reciprocal join showed evidence for the presence of D segments. To amplify this region they used a primer derived from the conserved DH recombination signal sequence and mbr or mcr bcl-2 primers. The 14q+ strand on the translocated chromosome was amplified as usual with a consensus JH primer again with mbr or mcr primers. It was possible in 2 patients to amplify both the join and reciprocal join, one with the mbr and one with the mcr primers. Comparing both sequences showed the N insertions in the 14q+ strand only and that they truly were non-template encoded nucleotides, with no bcl-2 deletion as previously reported (Bakhshi et al 1987). This indicated a similar mechanism of rearrangement for both the mbr and mcr loci. Sequence analysis of a series of follicular lymphomas with N insertions at the 14q+ locus demonstrated homology with a known DH segment in one patient, suggesting that in some cases long runs of N insertions may correspond to uncharacterised DH segments.

Booster PCR (Ruano et al 1989) involves performing two sets of amplification cycles initially with a low primer concentration, followed by a higher concentration to maximise yields. Price et al (1991a) used this technique in conjunction with nested PCR to amplify translocations from remission material to provide sufficient DNA to sequence. When compared to presentation tissue, 29% of patients demonstrated additional translocations which sequence analysis proved were novel and not due to carry over. These could represent secondary translocations by the initial clone, true biclonality or sporadic translocations occurring in normal B-lymphocytes. The final suggestion is supported by the evidence that t(14;18) translocations have been reported in reactive tissues which do not progress to neoplasia (Grace et al 1989) and in normal PB lymphocytes (Limpens et al 1992). This phenomenon may also explain the discovery of the t(14;18) in 30% of Hodgkin's lymphoma patients (Stetler-Stevenson et al 1990) which may derive from normal B-lymphocytes rather than Reed-Sternberg cells.

A possible mechanism mediating the translocation event was proposed by Wyatt et al (1992) after more extensive sequence information was collated. They observed the tight clustering of bcl-2 breakpoints in 3 regions within a 150bp region of the mbr associated with short elements resembling prokaryotic chi recombination initiation

signals. The frequent observation of DXP sequences in excised signal joints lead them to suggest that the initial event in a translocation was a chi mediated bcl-2-DXP recombination followed by a DXP-JH recombination resolved by the regular V-D-J recombinase complex.

2.1.6 Aims

We wished to study and evaluate the t(14;18) as a potential tumour marker which could be used to monitor minimal residual disease during remission in patients with low grade NHL and Hodgkin's lymphoma. Initially, to determine gross tumour infiltration, tumour samples were screened by Southern blot analysis with an IgHJ probe. PCR techniques to amplify the t(14;18) were evaluated and modified to improve their specificity in detecting the presence of minimal tumour. This modified PCR technique was then used to analyse our patients for the presence of this clonal marker. To confirm the involvement of bcl-2 and JH sequences in our PCR amplification products and to determine the position of the breakpoint within the mbr region PCR products were sequenced directly. From the sequence information the clustering of breakpoints and JH segment usage was analysed. On the basis of these studies we intended to monitor tumour contamination in patients undergoing standard chemotherapy and intensive myeloablative therapy.

2.2 PATIENTS

2.2.1 Patients

A total of 23 low grade NHL patients (15 male, 8 female) with a mean age of 54 years (range 32 to 79 years) were available for analysis. In addition 8 patients with Hodgkin's lymphoma (7 male, 1 female) with a mean age of 35 years (range 15 to 48 years) were studied.

2.2.2 Cytogenetic analysis

Cytogenetic analysis was carried out on the NHL patients by standard G-banding techniques on tumour samples of lymph node. Cytogenetic information was available on 14 patients indicating the presence of the t(14;18)(q32;q21) chromosome in 11, a further 2 with a t(8;14) translocation and one with a complex karyotype.

2.2.3 Cell lines

The SU DH L6 line (Epstein 1978) was provided by Dr. J. Cossman. It was derived from a peritoneal effusion from a 43 year-old male patient with a diffuse histiocytic lymphoma and carries the t(14;18)(q32;q21) translocation. The line was received as pellets frozen in DMSO and was cultured in IMDM with 10% FCS before DNA was extracted. The promyelocytic HL60 cell line was obtained commercially from ATCC and used as a source of control DNA with no t(14;18) translocation.

2.3 MATERIALS

DNA extraction

Ficoll-paque (density 1.077g/ml) (Pharmacia, Uppsalla, Sweden)

Saline solution

NaCl	0.85%

Frozen section lysis solution

Sucrose	0.32M
Tris pH7.5	0.01M
$MgCl_2$	0.005M
Triton-X 100	1%

Frozen section nuclear lysis solution

NaCl	0.075M
EDTA	0.024M

High TE

Tris HCl pH7.6	0.1M
EDTA pH8.0	0.04M

Lysis solution

Tris HCl pH7.6	0.1M
EDTA pH8.0	0.04M
SDS	0.2%
NaCl	1.0M

TE pH7.6

Tris HCl pH7.6	0.01M
EDTA pH8.0	0.001M

RNase A (Sigma, Poole, Dorset, U.K.) 5mg/ml, boiled 10min

Proteinase K (Sigma) 10mg/ml

Tris buffered phenol (Camlab, Cambridge, U.K.)

Equilibrated to pH8.0 (aqueous phase) in buffer supplied

Gel electrophoresis loading buffer

Stock solution 6x concentrated

Glycerol	5.0%
Bromophenol blue	0.041%
Xylene cyanol	0.041%

Tris acetate buffer (TAE)

Stock solution 20x concentrated

Tris-acetate	0.04M
EDTA	0.001M

Tris borate buffer (TBE)

Stock solution 10x concentrated

Tris-borate	0.089M
EDTA	0.002M

TEMED (Sigma)

38% acrylamide 2% bisacrylamide (NBL, Cramlington, U.K.)

SeaKem LE agarose (Flowgen, Sittingbourne, Kent, U.K.)

Electrophoresis grade

SeaKem GTG agarose (Flowgen)

Genetic technology grade, low melting point

NuSieve 3:1 agarose (Flowgen)

Electrophoresis grade

NuSieve GTG agarose (Flowgen)

Genetic technology grade, low melting point

Ethidium bromide (Sigma)	10mg/ml

Southern blotting

Acid hydrolysis

HCl	0.25M

Denaturing solution

NaCl	0.6M
NaOH	0.4M

Neutralising solution

NaCl	1.5M
Tris	0.5M

SSC

Stock Solution 20x concentrated	
NaCl	0.15M
Tri Sodium Citrate	0.015M

Hybridisation

Hybridisation buffer

NaH_2PO_4 pH7.5	0.25M
EDTA pH8.0	0.01M
Bovine serum albumin (Sigma)	1.0%
SDS	7.0%

Phosphate buffer

NaH_2PO_4	1.0M

Adjust to pH7.5 with Na_2HPO_4

Random primed DNA labelling kit (Boehringer Mannheim, Germany)

Klenow polymerase buffer

Stock solution 10x concentrated	
Potassium phosphate	0.04M
$MgCl_2$	0.0066M
β-Mercaptoethanol	0.0001M

Nick column (Pharmacia)

dCTP ^{32}P (DuPont Nemours, Dreieich, Germany)

Wash solution 1

SSC	2x

Wash solution 2

SSC	2x
SDS	1.0%

Wash solution 3

SSC	0.1x
SDS	0.1%

Developer (Sigma)

GBX developer	20 %

Fix (Sigma)

GBX fixer and replenisher	20%

Stop (Sigma)

Indicator stop bath	4%

X-ray film Hyperfilm MP (^{32}P emission), hyperfilm βmax (^{35}S emission) Amersham International, Amersham, Buckinghamshire, U.K.

Nylon membranes GeneScreen Plus (DuPont)

Probe stripping solution

Tris pH7.6	0.01M
EDTA pH8.0	0.001M
SDS	1%

Polymerase chain reaction buffer

Stock solution 10x concentrated

Tris HCl pH8.3	0.01M
KCl	0.05M
Gelatin	0.01%

dNTP solution

Stock Solution 10x concentrated

dATP	0.2mM
dCTP	0.2mM
dGTP	0.2mM
dTTP	0.2mM

Mineral oil (Sigma)

Taq DNA polymerase (Advanced Biotechnologies, London, U.K.)

Slot blotting hybridisation

Denaturing solution

NaOH	0.4M
EDTA pH8.0	0.025M

Recovery of DNA fragments from agarose gels

Gene-Clean II kit (Stratech Scientific, Luton U.K.)

MERmaid kit (Stratech)

DNA sequencing

Sequenase DNA sequencing kit (USB, Cambridge, U.K.)

Repel Silane (Pharmacia)

Bind Silane (Pharmacia)

dATPα^{35}S specific activity >1000 Ci/mmol (DuPont)

Synthetic oligonucleotides (Oswell DNA Services, Edinburgh U.K.)

Obtained in dH_2O, after gel filtration.

All chemicals were of Analar grade and supplied by BDH (Poole, Dorset, U.K.) unless otherwise stated.

Restriction endonucleases supplied by Promega limited (Southampton, U.K).

2.4 METHODS

All methods are based on Sambrook, Fritsch and Maniatis (1989) unless otherwise stated.

2.4.1 DNA extraction

2.4.1.1 Peripheral blood and bone marrow

PB or BM collected at presentation or in the course of therapy was layered onto Ficoll-Paque and centrifuged at

400g for 20min. MNC were suspended in high TE and lysed with an equal volume of lysis solution. RNase A was added to a final concentration of 50μg/ml before incubation at 37°C for 30min. Proteinase K was added to a final concentration of 100μg/ml before incubation at 40°C for 16h. Lysates were then mixed on ice with an equal volume of tris- buffered phenol and the aqueous phase collected by centrifugation at 1600g for 10min. This was repeated until the aqueous phase appeared clear at which stage extraction with an equal volume of chloroform/isoamyl alcohol was performed.

DNA was precipitated by the addition of 0.5 volumes of 7.5M ammonium acetate and 2 volumes of absolute ethanol before washing with 66.7% ethanol. After drying for 5min the DNA was resuspended in 500μl of TE and left to dissolve at 4°C for several days. If no precipitate formed, the sample was transferred to -40°C overnight and the DNA collected by centrifugation at 1600g for 30min at 4°C. The pellet was resuspended in 1ml 70% ethanol, further centrifuged at 16000g for 10min at 4°C and dried under vacuum. Finally, the DNA was resuspended in 100μl TE.

When dissolved, an aliquot of the sample was diluted 1:100 in dH_2O to enable the concentration and purity to be measured by UV spectroscopy. Absorbance readings were taken at 260 and 280nm, 1.0 OD unit at 260nm being equivalent to 50ng of DNA. An OD_{260}:OD_{280} ratio in the

1.8-2.0 range indicated sufficient purity.

2.4.1.2 Mononuclear cells

MNC were isolated from PBSC harvests by Ficoll-hypaque density gradient centrifugation (400g for 30 min) and collected in culture medium. Cells were pelleted at 700g for 5min, suspended in an equal volume of TE and lysed with an equal volume of lysis solution. The lysates were processed as detailed above.

2.4.1.3 Biopsy material

Such material, collected at presentation or relapse consisted of lymph node, skin or spleen and was obtained as fresh tissue. Following maceration with a razor blade, the biopsy specimen was suspended in high TE and an equal volume of lysis solution added before the DNA was extracted as above.

2.4.1.4 Frozen sections

Fresh frozen cryostat sections 10μm thick were lysed according to the method of Cotter et al (1988). Approximately 20 sections were suspended in 10ml lysis solution at 4°C then centrifuged at 700g for 20min. The pelleted nuclei were resuspended in 2.5ml nuclear lysis solution with 125μl 10% SDS and 50μl proteinase K and incubated overnight at 37°C. Phenol/chloroform extraction and ethanol precipitation were performed as above before dissolving the DNA in 100μl TE.

2.4.2 Restriction endonuclease digestion

To detect the alteration in restriction enzyme sites caused by translocation or rearrangement events, 7μg of genomic DNA was mixed with digestion buffer to a final concentration of 1x and dH_2O to a volume of 68μl. Samples were mixed on ice for several hours before the addition of 35U of HindIII or BamHI. After gentle mixing the reactions were transfered to 37°C for 30min followed by the addition of a further 35U of enzyme. Digests were performed for a further 16h.

2.4.3 Southern blotting

2.4.3.1 Gel electrophoresis

For size fractionation of restriction enzyme digests, 0.8% SeaKem LE agarose gels were cast in 1x TAE. Gels were submerged in 1x TAE buffer and samples loaded in 1x loading buffer. Electrophoresis was performed for 16h at 2V/cm. Bacteriophage lambda DNA, digested with HindIII was included as a size marker. Staining was performed in 1x TAE with ethidium bromide at 0.5μg/ml and viewed under UV light.

2.4.3.2 Transfer of DNA to nylon membranes

DNA was transfered to nylon membranes by the method of Southern (1975). Gels were acid hydrolysed, denatured then neutralised in the relevant solutions for 30min each step. The gel was placed on a wick of Whatman N°6 3mm filter paper and immersed in 10x SSC. A sheet of nylon membrane previously soaked in 10x SSC was laid on top of

the gel, followed by 3 sheets of filter paper, also pre-soaked in 10x SSC. A layer of paper towels approximately 10cm thick was placed on top of the filter paper followed by a glass plate and weight. Capillary transfer was allowed to occur for 16h before the membranes were removed, rinsed in 2x SSC before air drying.

2.4.3.3 Probe

To detect the presence of IgHJ rearrangements, a joining region probe (Flannagan and Rabbitts 1982) consisting of a 2.5kb fragment maintained in the SacI site of a pAT plasmid (Figure 2.1) was used. The probe was prepared by digestion of the plasmid with SacI followed by electrophoresis through 0.8% SeaKem GTG agarose (section 2.4.3.1). The relevant band was excised with a new razor blade and purified by extraction with glass beads (section 2.4.6.1).

Labelling was performed according to the method of Feinberg and Vogelstein (1983) to a specific activity of $>10^9$cpm/μg DNA. To 25μg probe was added buffer/ hexanucleotide mix to a final concentration of 1x, 0.5M nucleotide mix (dATP, dGTP, dTTP), 2U Klenow fragment DNA polymerase I and 30μCi a^{32}P dCTP. The reaction was incubated overnight at room temperature before incorporation was calculated by the precipitation of incorporated nucleotides on to Whatman DE81 filters with 0.5M phosphate buffer pH7.0. Unincorporated nucleotides specific activity of the eluted fraction determined by

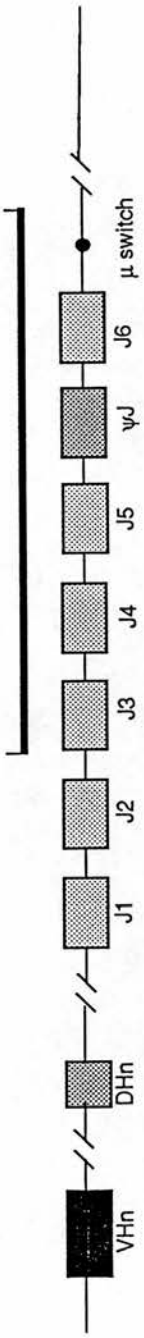


FIGURE 2.1 Diagram of IgH locus showing location of J segments. The location of IgHJ probe used in Southern blotting is indicated.

counting an aliquot in a β-scintillation counter.

2.4.3.4 Hybridisation

Incubation steps were performed in a dedicated hybridisation oven (Robbins Scientific) in the tubes supplied. Membranes were rolled, placed inside the hybridisation tubes and sufficient hybridisation fluid added to allow even wetting. A further 3ml of fluid was added and the tubes placed in the oven at 65°C. The probe was denatured by boiling and plunging on ice before sufficient probe solution to give $4.5x10^6$ dpm total ($1.5x10^6$dpm/ml) was injected into the tube, taking care not to touch the membrane with neat probe. Membranes were returned to the oven and allowed to rotate overnight.

2.4.3.5 Washing

The excess fluid was poured off, 20ml of wash 1 added and the tube rotated at room temperature for 30min before the solution was replaced with wash 2. Washing was continued at 65°C for 30min before the addition of wash 3 and incubation at room temperature for a further 30min. Radioactivity was monitored with a hand held counter, when a reading of 2-5cps was obtained the membrane was removed and wrapped in cling film otherwise wash 3 was repeated at 65°C until the radioactivity was reduced sufficiently.

2.4.3.6 Autoradiography

Membranes were placed in an X-ray cassette next to

preflashed X-ray film and exposure performed at -70°C for one to 14d. The film was developed by immersion in developer solution for 5min, stop solution for 30s and finally fixer for 2min before rinsing in running water for 5min and drying.

2.4.4 Polymerase chain reaction analysis

The initial PCR protocol followed was that described by Stetler-Stevenson et al (1988) with some modification. Amplification was performed with the bcl-2(25mer) and J1,2,4,5; J3 and J6 primers (Table 2.1) which delineate a product of 150-300bp. The number of cycles was reduced as it had been suggested that yields decrease if more than 30 cycles were performed (Sambrook et al 1989) and a final elongated extension step was included to increase yields. Various optimisation steps were performed to improve specific product yield and sensitivity.

2.4.4.1 Reaction conditions

Each 100μl reaction contained 1x PCR reaction buffer, 200μM each dNTP, 1.5mM $MgCl_2$, 500ng each primer and 2.5U Taq polymerase. Cycles were as described in Table 2.2 and carried out in a thermal cycler (Hybaid Limited, Teddington, Middlesex U.K.). In order to optimise the yield of the specific product, several parameters were tested. Initially the annealing and extension conditions were altered to improve the specificity, followed by primer and magnesium ion concentration titrations using 1μg SU DH L6 DNA as the template in each instance.

Oligo	Sequence	Tm(°C)
bcl-2 (25mer)	5'-TTA GAG AGT TGC TTT ACG TGG CCT G-3'	74
IgHJ 1,2,4,5	5'-GGA CTC ACC TGA GGA GAC GGT GAC C-3'	82
IgHJ 3	5'-CAT CTT ACC TGA AGA GAC GGT GAC C-3'	76
IgHJ 6	5'-AAA CAA AGG CCC TAG AGT GGC CAT T-3'	74
bcl-2 (20mer)	5'-TTG ACC TTT AGA GAG TTG CT-3'	56
IgH 1-6	5'-ACC TGA GGA GAC GGT GAC C-3'	62
Internal bcl-2 probe	5'-CAA CAC AGA CCC CAG AGC CCT CCT GCC CTC CTT CCG CGG GGG C-3'	
Labelling Hexamer	5'-GCC CCC-3'	

Table 2.1 t(14;18) major breakpoint cluster region (mbr) oligonucleotide sequences.

Temperature (°C)	Time (s)	Number Of Cycles
94	420	1
55	120	
Taq polymerase added		
74	120	
94	120	28
55	120	
74	180	
94	120	1
55	120	
74	600	

Table 2.2 Initial PCR cycling conditions, bcl-2(25mer), J1,2,4,5; J3; J6 amplimers.

2.4.4.2 Extension time

Two extension times (180s and 120s) were analysed (Figure 2.2, lanes 2 and 3 respectively) and an extension time of 120s resulted in a reduction of non-specific product bands and was used in all further amplifications.

2.4.4.3 Annealing time

Two annealing times (120s and 90s) were analysed (Figure 2.2, lanes 3 and 4). A 60s annealing time further reduced the presence of non-specific PCR bands and was used in all subsequent amplifications.

2.4.4.4 Primer concentration

The effect of primer concentrations of 500, 250 and 125ng per reaction are shown in Figure 2.2 (lanes 4, 5 and 6). Analysis of the PCR product demonstrated maximum yields using 125ng (Figure 2.2 lane 2) of each amplimer and this concentration was used in subsequent amplifications.

2.4.4.5 Magnesium concentration

The concentration of magnesium ions was increase from 1.0 to 3.5mM in 0.5mM increments (Figure 2.2, lanes 7 to 12) with 1.5mM generating the maximum target band and minimal non-specific banding. However 2 bands were still consistantly obtained and titration using 0.25mM increments from 0.5 to 1.5 mM was performed (Figure 2.3, lanes 2 to 6). Two bands were consistantly observed in each lane due to mispriming, possibly mediated by the homology of the 3'- primers with each JH segment (Figure

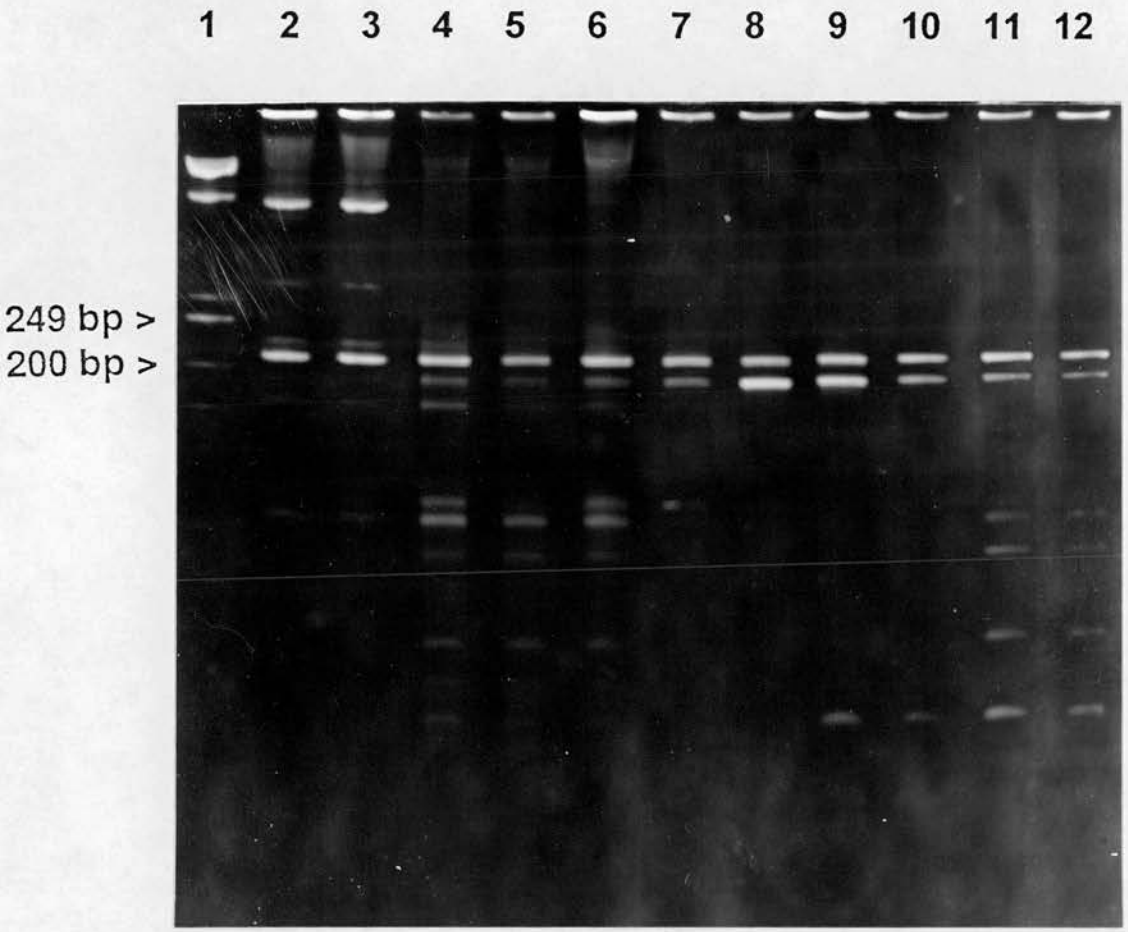


Figure 2.2 Ethidium bromide stained 5% PAGE of PCR amplification products of SUDHL6 DNA. Lane one contains ϕX174 DNA digested with Hinf1 (band sizes 726, 713 553, 500, 427, 417, 413, 311, 249, 200, 151, 140, 118, 82, 66, 48, 42, 40, 24bp). Amplification was carried out using cycle conditions in Table 2.2 with the following modifications to optimise specificity. Lanes 2 and 3 compare extension times of 180s and 120s. Using 120s extension time, lanes 3 and 4 compare annealing times of 120s and 60s. Using 120s extension and 60s annealing, lanes 4, 5 and 6 compare 500, 250 and 125ng primer concentrations. Using 125ng of primer, lanes 7 to 12 show the effects of increasing magnesium concentrations from 1.0 to 3.5mM in 0.5mM increments.

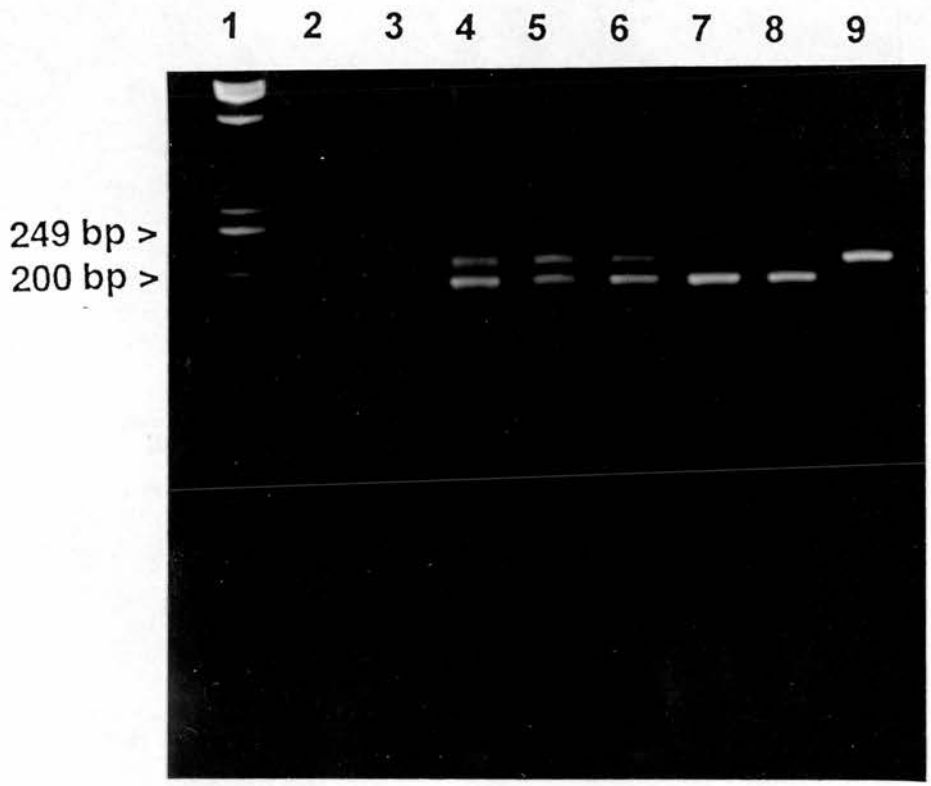


Figure 2.3 Ethidium bromide stained 5%PAGE of PCR amplification products of SUDHL6 DNA. Lane 1 contains ϕX174 DNA digested with HinfI. Lanes 2 to 6 show the effects of increasing magnesium concentration of 0.5 to 1.5mM in 0.5mM increments. Using 1.5mM magnesium concentrations lanes 7-9 show the amplification products of each individual 3' amplimer used in conjunction with the 5' bcl-2 amplimer. Amplificaton in lane 7 was carried out with 3' amplimer IgHJ1,2,4,5; lane 8 IgHJ3 and lane 9 IgHJ6.

Figure 2.4 Diagram of the IgH locus joining segment J1 to J6. The binding site for amplimer J1,2,4,5;J3 and J6 is shown on each J segment. A 19base region of homology to all 6 segments if boxed.

2.4). The cell line SU DH L6 must have J6 juxtaposed to Bcl-2 and J6 has two possible amplimer binding sites as shown in Figure 2.4, explaining the consistent appearance of two bands. To test this a reaction using each individual 3'- primer in conjunction with the 5' bcl-2 amplimer was performed (Figure 2.3 lanes 7 to 9) resulting in a single band in each lane, with a heavier band apparent in the J6 amplimer lane corresponding to the more 3' binding region. A further magnesium titration is shown in Figure 2.5 with concentrations in lanes 2 to 10 of 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0mM $MgCl_2$ performed with the bcl-2 and J1,2,4,5 amplimer. A single discrete band can be seen in the 2.0mM lane.

2.4.4.6 Cycle number

As amplification of the template is exponential only for part of the PCR process, a cycle number titration was performed to maximise yields of specific product. Figure 2.6 shows duplicate reactions removed from the heat block between 25 and 34 cycles (lanes 1-10). Analysis of the PCR product on PAGE demonstrates optimal yields with 31 cycles of amplification.

2.4.5 Analysis of PCR products

2.4.5.1 Polyacrylamide gel electrophoresis

For fine resolution of PCR products, 5% vertical PAGE gels were cast in 1x TBE buffer and samples loaded in 1x loading buffer. Electrophoresis was carried out at 10V/cm for 90min with HinfI digested X174 DNA, or occasionally

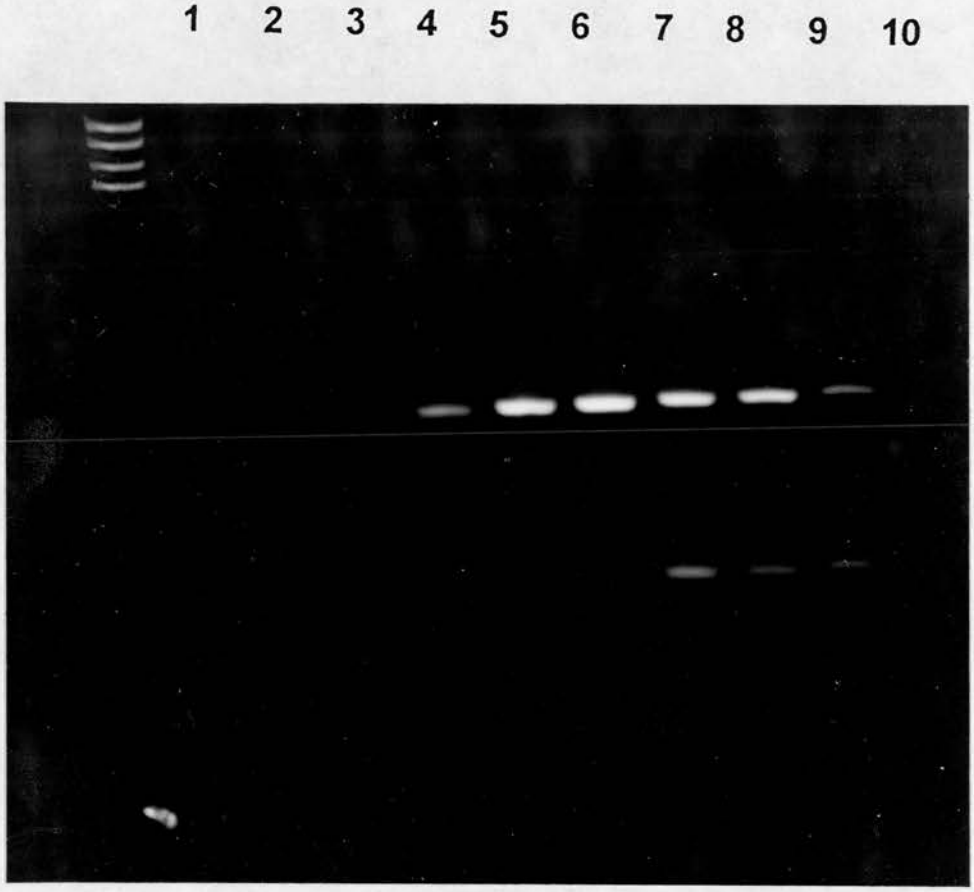


Figure 2.5 Ethidium bromide stained 5%PAGE of PCR amplification products of SUDHL6 DNA. Lane 1 contains bacteriophage lambda DNA digested with EcoRI and BamHI. Amplification was performed with the amplimers bcl-2 and J1,2,4,5. Lanes 2-10 show the effects of increasing magnesium concentrations of 0.6, 0.8, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0 and 6.0 mM.

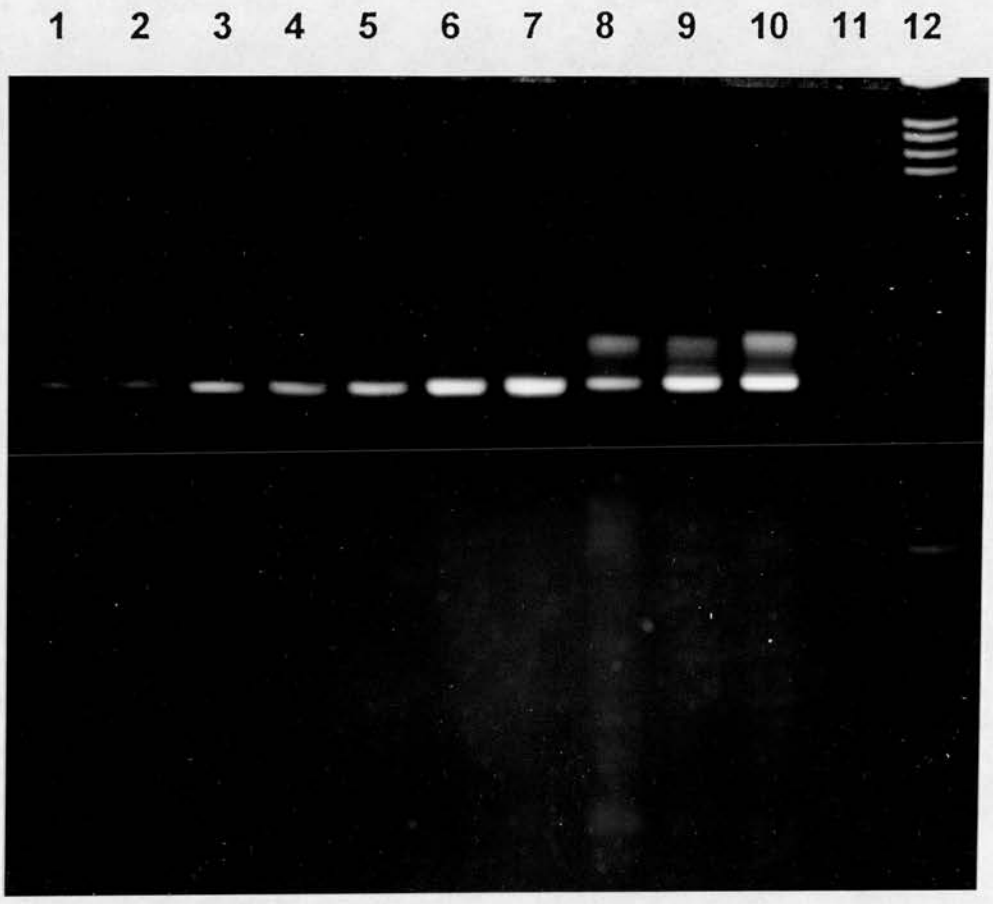


Figure 2.6 Ethidium bromide stained 5%PAGE of PCR amplification products of SUDHL6 DNA. Lane 12 contains bacteriophage lambda DNA digested with EcoRI and BamHI. Lanes 1 to 10 show the effects of increasing the number of amplification cycles from 25 (lane 1) to 34 (lane 10) in 1 cycle increments.

bacteriophage lambda DNA digested with HindIII and BamHI, as molecular weight size markers. Gels were stained for 15min in 1x TBE with ethidium bromide at 0.1μg/ml, rinsed in dH_2O then viewed under UV.

2.4.5.2 Agarose gel electrophoresis

This was performed as described in section 2.4.4.1 using 4% NuSieve 3:1 agarose and HinfI digested ϕX174 DNA size markers.

2.4.5.3 Slot blotting

To 2μl of PCR product was added 98μl denaturing solution before boiling for 3min. After incubation on wet ice for 2min samples were slotted onto a nylon membrane pre-soaked in 20x SSC using a slotblot manifold (BRL Hybrislot). Vacuum was applied until the wells had drained, the vacuum shut off and 50ml of 2x SSC added and the vacuum replaced until all the fluid had drained. The membrane was then removed and immersed in 2x SSC before drying. Membranes were stored in cling film.

2.4.5.4 Southern blotting

Following agarose gel electrophoresis as described in section 2.4.5.2, capillary transfer was performed as described in section 2.4.3.2 with the acid hydrolysis step omitted and denaturation and neutralisation steps reduced to 15min each.

2.4.5.5 Probes

The IgHJ probe was as described in section 2.4.3.3. In addition, a bcl-2 oligonucleotide probe derived from a region 5' of the mbr (Stetler-Stevenson et al 1988) (Table 2.1) was used. The bcl-2 probe was labelled as described for the JH probe above except the buffer/ hexanucleotide mix primer solution was substituted with Klenow polymerase buffer and 100pM of a specific hexanucleotide complementary to the 3' end of the probe replaced the random hexanucleotides (Table 2.1)

2.4.5.6 Hybridisation

Hybridisation steps were performed as 2.4.3.4 for 1h only.

2.4.5.7 Washing

Membranes were rinsed in wash 1 at room temperature for 15min before stringent washing in wash 3 at 65°C for 30min.

2.4.5.8 Autoradiography

With the exception of shorter exposure times of 2 to 16h, this was carried out as detailed in section 2.4.3.6.

2.4.6 Direct sequencing

2.4.6.1 DNA purification

Mineral oil was removed from PCR reactions with 3 volumes of chloroform, followed by the addition of 3 volumes of sodium iodide and by 5μl of vortexed glass beads. After

incubation at room temperature for 5min, the sample was pulse spun at 16000g, the supernatant discarded and the pellet washed 3 times in ethanol wash. Purified DNA was eluted into 10μl of dH_2O by incubating for 5min at 55°C followed by a pulse spin. The elution step was repeated and the eluates pooled.

2.4.6.2 Sequencing reaction

Sequencing was based on the dideoxy chain termination method (Sanger et al 1977) with the modifications of Winship (1989). PCR products were sequenced in both directions using the PCR amplimers as sequencing primers. The annealing reaction contained 40mM Tris HCl pH7.5, 25mM $MgCl_2$, 50mM NaCl, >50pmol primer, 10% DMSO and approximately 100ng template DNA in a volume of 10μl. This reaction was boiled for 3min, plunged into methanol at -70°C for 10s before transfer to wet ice. This was mixed with the labelling reaction which contained 0.025M DTT, 0.5μM dCTP, 0.5μM dGTP, 0.5μM dTTP, 5μCi a^{35}S dATP and 2U sequenase enzyme in a total volume of 6μl. Immediately 3.5μl of the resulting mix was added to 2μl of each termination reaction preheated to 50°C. These consisted of 80μM dATP, 80μM dCTP, 80μM dGTP, 80μM dTTP and 10% DMSO. The "A" reaction also contained 8μM ddATP, the "C" reaction 8μM ddCTP, the "G" reaction 8μM ddGTP and the "T" reaction 8μM ddTTP. The termination reaction was allowed to continue for 3min before the addition of 4μl stop solution containing 95% formamide, 20mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol. Completed

reactions were stable for several days at -20°C before electrophoresis.

2.4.6.3 Electrophoresis

Samples were heated to 80°C for 3min prior to electrophoresis through 0.2mm thick, 5% PAGE gels cast in 1x TBE with 8M urea. A Pharmacia LKB sequencing rig was employed and gels run at a constant temperature of 55°C at 30V/cm for 2h. At the end of electrophoresis, the gel was washed twice in 10% acetic acid for 10min and dried onto the glass sequencing plate.

2.4.6.4 Autoradiography

Dried gels were exposed to Hyperfilm βmax X-ray film (not pre-flashed) at room temperature for one to 7d before developing as described in section 2.4.3.6 with the fix step extended to 5min.

2.4.6.5 Data analysis

Mbr junction nucleotide sequences were aligned with published bcl-2, JH and DH segments (Ravetch et al 1991, Ichihara et al 1988) using the University Of Wisconsin Genetic Computer Group (UWGCG) software package.

2.4.7 Design of alternative amplimers

Due to the non-specific nature of the 3' JH amplimers, a single consensus 3' amplimer was designed. Using the GAP program through UWGCG, a 19bp consensus region was identified (Figure 2.4). In addition a new 5' mbr bcl-2 amplimer was selected to ensure a matched annealing temperature. This was chosen from a region immediately 5' to the existing primer to generate a product of a similar size to the previous amplimer set. The 5' amplimer was designed according to the following criteria (Innis and Gelfand 1990):-

a) No 3' end complementarity between the amplimers in order to avoid primer-dimer artifacts.

b) Select a region with average GC content.

c) Avoid extensive runs of G or C nucleotides.

With these constraints taken into consideration, a 20bp primer was synthesised (Table 2.1).

2.4.7.1 Reaction conditions

The reaction conditions were as described in section 2.4.4.1 except that 250pmol of each primer was used. The cycling conditions are shown in Table 2.3.

2.4.7.2 Optimisation

A magnesium titration was performed using 0.5 to 3.0mM $MgCl_2$ in 0.5mM increments (Figure 2.7, lanes 2-7) with the optimal result seen in lane 4, 2.5mM.

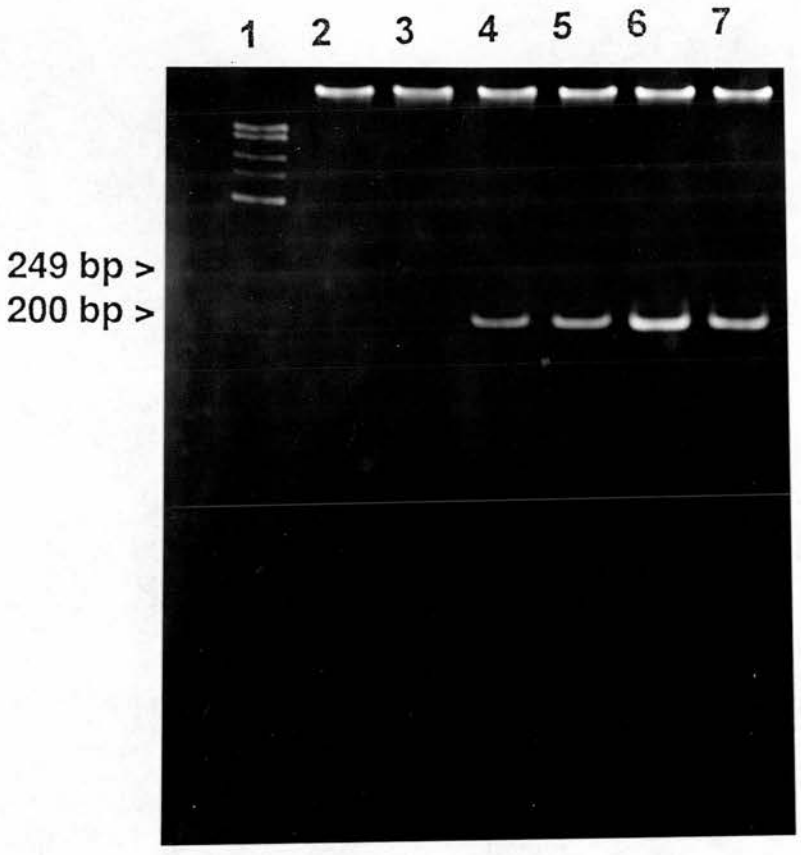


Figure 2.7 Ethidium bromide stained 5%PAGE of PCR amplification products of SUDHL6 DNA. Lane 1 contains ϕX174 DNA digested with HinfI. Using the Bcl-2 (20mer) amplimer and the IgHJ1-6 consensus amplimer (Table 2.1) and cycling conditions shown in table 2.3. Lanes 2 to 7 show the effects of increasing magnesium concentration of 0.5 to 3.0mM in 0.5mM increments.

2.4.7.3 Prevention of false positivity

The extreme sensitivity provided by PCR can also lead to the amplification of products from previous reactions giving false positive results. The effect of contamination can be minimised by the careful separation of pre amplification reagents and post amplification products. The steps recommended by Kwok and Higuchi (1989) were taken and consisted of the use of separate laboratories for preparation and analysis of PCR reactions, storage of reagents in small single use aliquots, the use of aerosol resistant pipette tips and negative control reactions which contained no template DNA to monitor "carry-over".

2.4.7.3 Conclusion

The use of multiple 3'- primers does produce specific amplification but the use of a single consensus amplimer results in a single PCR product making interpretation of results less ambiguous. Resolution of two closely spaced bands in agarose is difficult, making sequencing from the 3'- amplimer difficult if J6 was involved. This problem is obviated by the use of a single amplimer. In addition the use of several 25mer amplimers is expensive compared to the use of a single pair of shorter oligonucleotides also simplifying the preparation of reactions.

2.5 RESULTS

2.5.1 Southern blotting

The sensitivity of Southern blotting in detecting clonal populations of neoplastic cells varies depending on the hybridisation characteristics of the probe used and the quality of the DNA studied, but is generally at the one to 5% level (van Dongen and Wolvers-Tettero 1991).

The JH probe is derived from the 3'region of the IgHJ locus and will generate a band of 9.5kb when hybridised against HindIII or BamHI digested genomic DNA with the IgHJ region genes in germline, or non-rearranged configuration. If a significant population of neoplastic cells are present (i.e. >5%) then a band of a different size will be apparent due to the alteration in pattern of restriction enzyme sites generated by a rearrangement. When a monoallelic rearrangement is involved, a single new band will be seen along with the germline band derived from the non-rearranged chromosome and any non-neoplastic cells in the sample. In cases where a biallelic rearrangement has occurred, two new bands will be seen in addition to any germline band contributed by normal cells.

A group of 19 patients with low grade NHL were studied. DNA from involved tumour tissue, lymph node in each case with the exception of bone marrow in patients JW and HG, was digested with the restriction enzymes HindIII or

BamHI, blotted and hybridised against the JH probe (Figure 2.8). Rearrangements were apparent in all 19 patients, with a biallelic pattern evident in 11. In addition lymph node DNA from all 8 patients with HD were digested and hybridised as described above, however only germline bands were observed.

All low grade patients studied therefore had appreciable degrees of tumour as demonstrated by Southern blotting (i.e. greater then 5%) but no clonal populations could be detected in the group with Hodgkin's lymphoma.

2.5.2 PCR initial primers

2.5.2.1 Sensitivity

The optimal PCR conditions were as follows: 1μg genomic DNA, 200μM each dNTP, 250ng each primer, 50mM KCl, 10mM Tris pH8.3 and 2.5mM $MgCl_2$. The cycling conditions are detailed in Table 2.3.

Tenfold dilutions of the t(14;18) cell line were made in the HL60 cell line, ranging from 10^{-1} (one tumour cell in a background of 10 HL60 cells) to 10^{-7} (one tumour cell in 10000000 HL60 cells) and the DNA extracted. PCR amplification was performed using the bcl-2 and J6 primers on 1μg of the mixed DNA along with a 100% SU DH L6 positive control and the products analysed by PAGE (Figure 2.9). Bands of the expected 237bp size can be seen in lanes 2 to 6, with no band evident in lane 7 (the 10^{-5} lane) indicating the limit of detection at

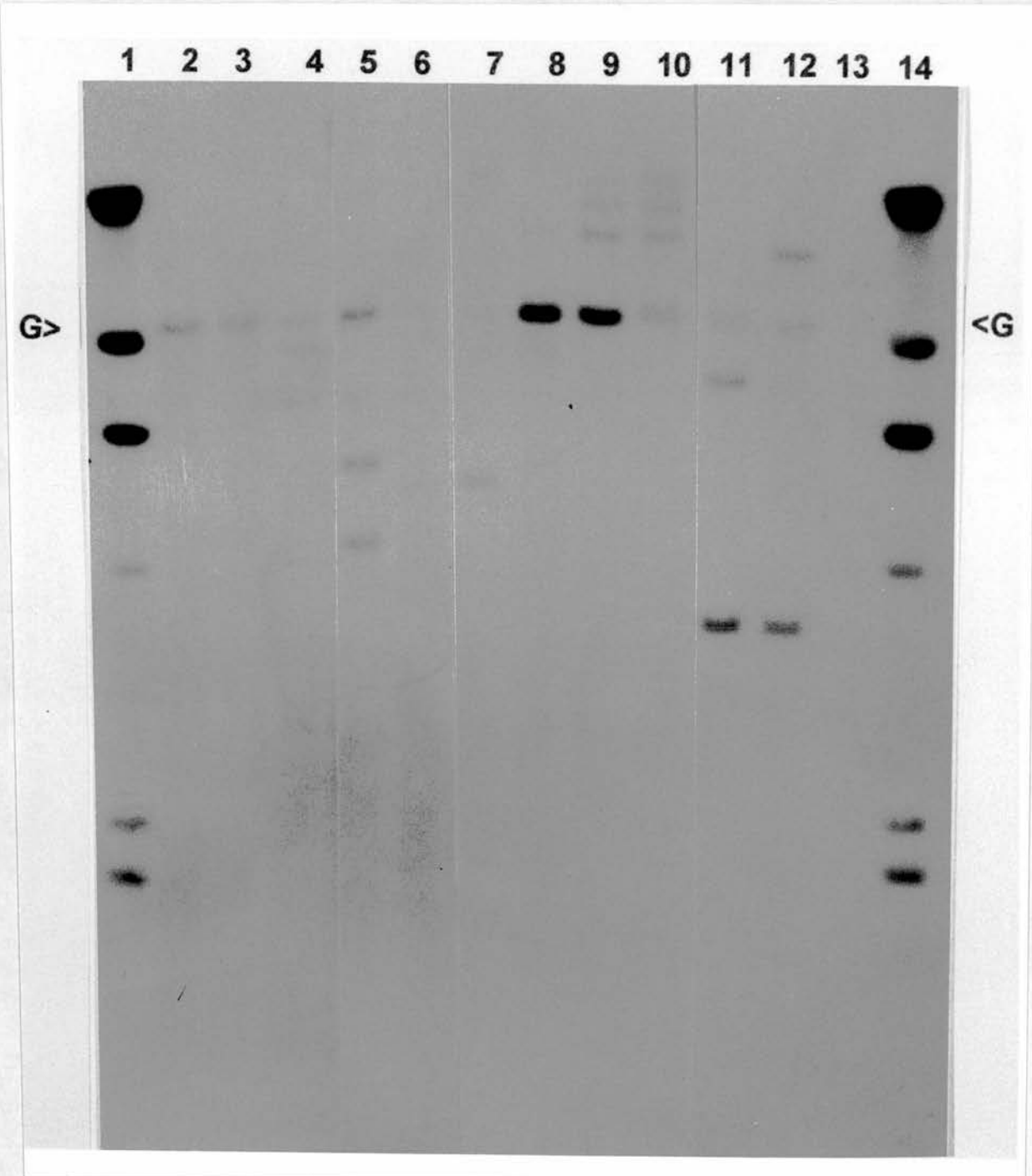


Figure 2.8 Autoradiograph of HindIII digested DNA from 10 patients with low grade NHL (lanes 2 to 12) hybridised against the JH probe. Lane 2 contains normal PB DNA and lane 13 no DNA. HindIII digested bacteriophage lambda DNA was run in lanes 1 and 14 (band sizes 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56kb). The location of the germline band is indicated with a G and rearranged bands rangeing in size from 4 to 9kb can be seen. The bands which appear heavier than germline in lanes 9,10 and 12 are HindIII partial digestion artefacts.

Temperature(°C)	Time(s)	Number Of Cycles
94	90	
55	90	30
74	120	
94	30	
55	90	1
74	420	

Table 2.3 Modified PCR cycling conditions.

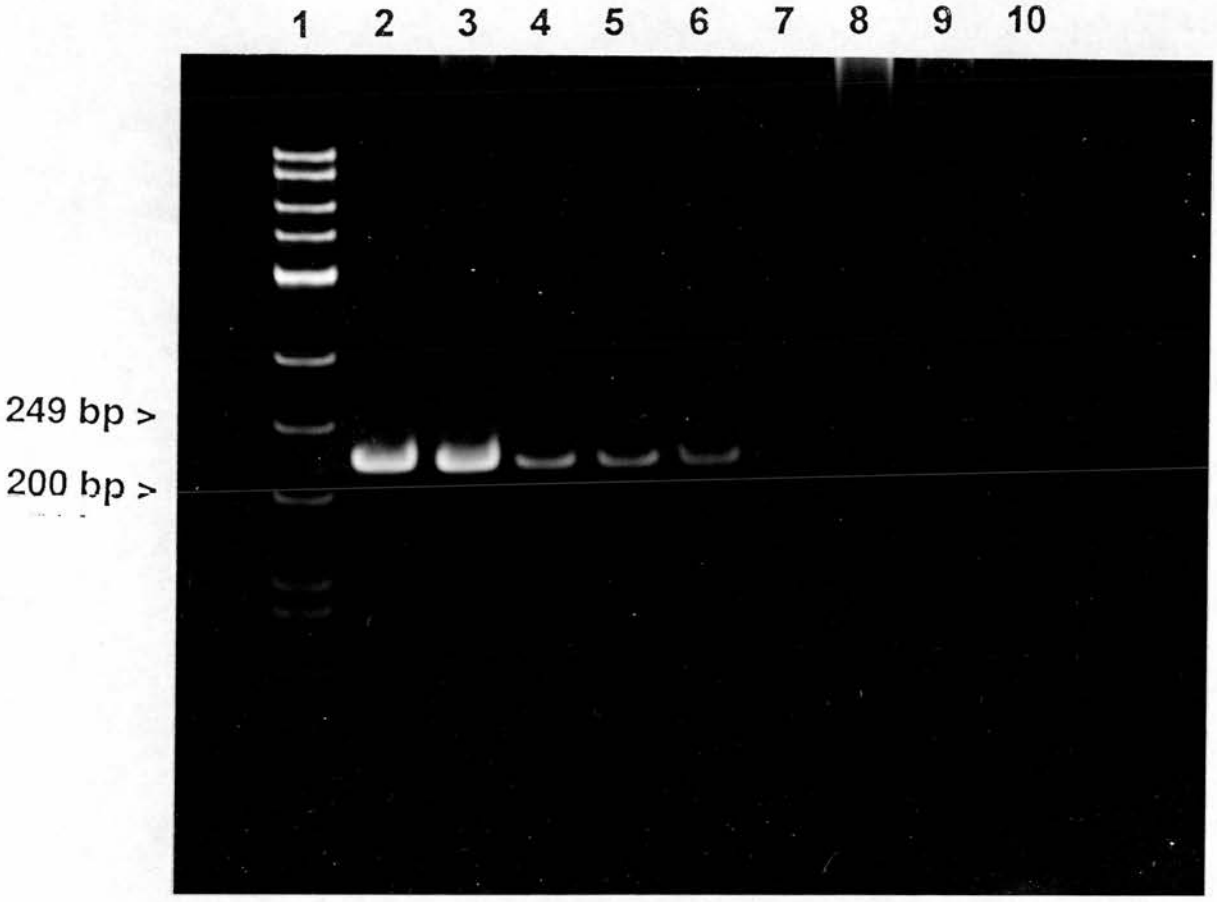


Figure 2.9 Ethidium bromide stained 5%PAGE of PCR amplification products of SUDHL6 DNA diluted in HL60 DNA. Lane 1 contains ϕX174 DNA digested with HinfI. Lanes 3 to 9 show tenfold dilutions of SUDHL6 amplified with the Bcl-2 (25mer) and J1,2,4,5 primers. Dilutions range from 10^{-1} to 10^{-7} with undiluted SUDHL6 DNA in lane 2. Lane 10 shows a negative control with no DNA template. The faint band observed in lane 8 did not subsequently hybridise to the bcl-2 probe.

one tumour cell in a background of 10000 normal cells. These products were slot blotted and hybridised against the bcl-2 probe (Figure 2.10) which facilitated a tenfold increase in sensitivity. Bands were evident in lanes 1 to 6 (the 10^{-5} lane) i.e. sensitivity at the one tumour cell in 100000 level. As one microgram of DNA was used as substrate, this represents the detection of a single copy of the bcl-2-JH sequence.

2.5.2.2 Substrate dependence

With only limited amounts of tissue available for analysis in some patients, the minimum PCR requirement for substrate DNA was investigated. To 4 reactions were added 10, 100 and 1000 whole cells which were be amplified directly without prior DNA extraction (Higuchi 1989) as well as purified DNA equivalent to 10000 cells (100ng). PAGE analysis was performed on the products (Figure 2.11) with no bands visible in lane 1, the control reaction with no DNA. Bands could be visualised in lanes 2 to 5 therefore DNA from 10 intact cells was sufficient to generate a detectable PCR product.

2.5.2.3 Screening

DNA prepared from presentation lymph nodes of 5 patients was amplified with the bcl-2 primer in conjunction with each 3'- JH primer in individual reactions. A PAGE gel was run (Figure 2.12) and distinct bands were visible in patients SP (lanes 2 to 4), LW (lanes 5 to 7), JI (lanes

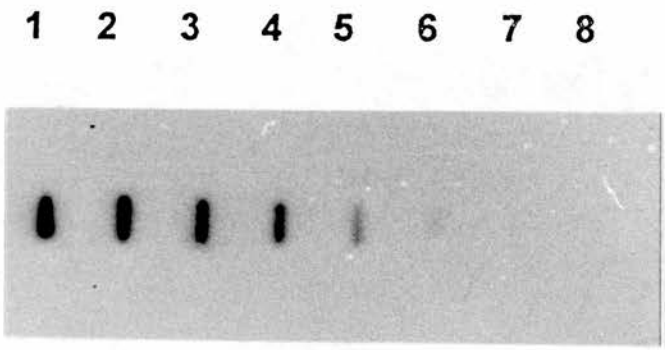


Figure 2.10 Autoradiograph of PCR amplification products of SUDHL6 DNA diluted in HL60 DNA and hybridised to the Bcl-2 probe. Slots 2 to 8 show tenfold dilutions of SUDHL6 amplified with the bcl-2 (25mer) and J1,2,4,5 primers. Dilutions range from 10^{-1} to 10^{-7} with undiluted SUDHL6 DNA in lane 1. Lane 8 shows a negative control with no DNA template.

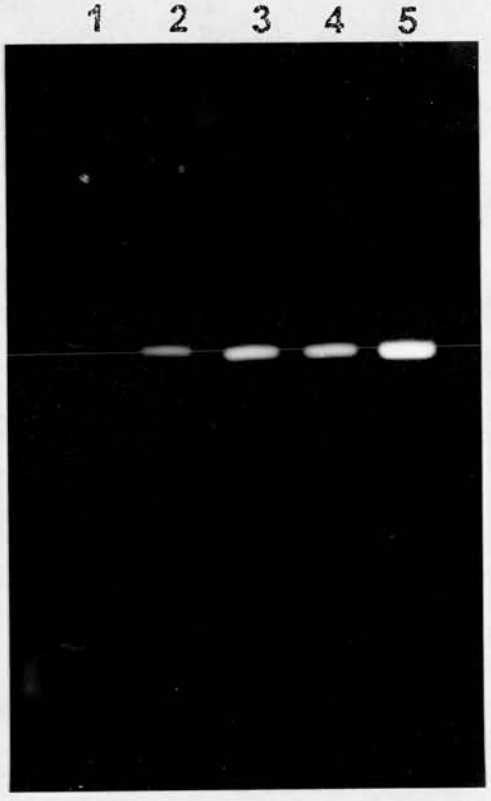


Figure 2.11 Ethidium bromide stained 5%PAGE of PCR amplification products of small number of whole SUDHL6 cells amplified with bcl-2 (25mer) and J1,2,4,5 amplimers. Lane 1 contains no template DNA, lanes 2 to 4 shows the amplification of 10, 100 and 1000 whole cells without prior DNA extraction. Lane 5 shows the amplification of 100ng of SUDHL6 DNA equivalent to 10 000 whole cells.

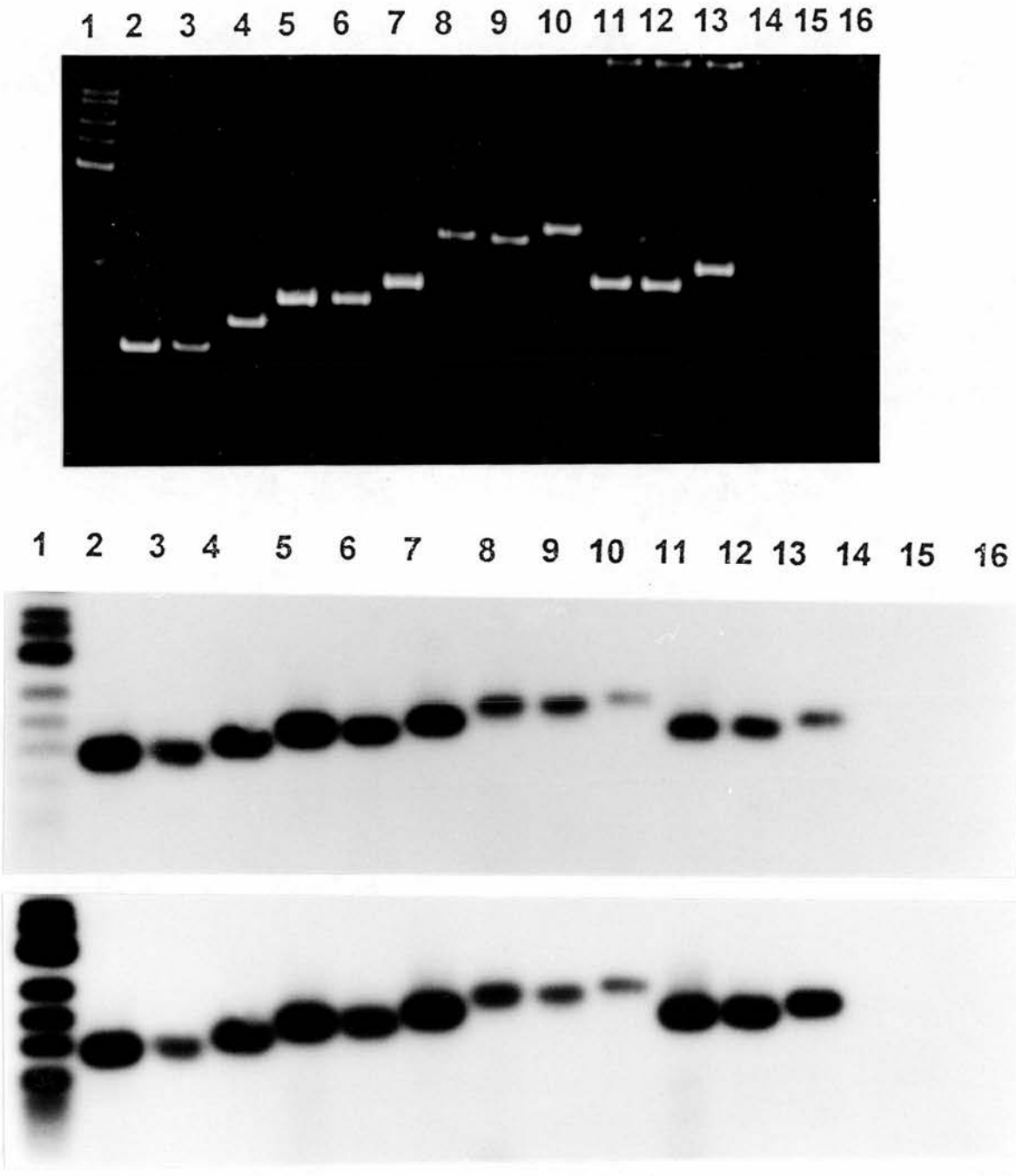


Figure 2.12 PCR amplification products of presentation lymph node DNA from 5 patients amplified with the bcl-2 (25mer) and each of the 3 individual IgHJ amplimers (Table 2.1). Lane 1 shows ϕX174 DNA digested with HinfI. Lanes 2-4 is amplified DNA from patient SP, lanes 5-7 patient LW, lanes 8-10 patient JI, lanes 11-13 patient JC and lanes 14-16 patient JK. The first lane from each patient is the amplificaton product using amplimer J1,2,4,,5; the second lane J3 and the third lane J6. The upper figure shows an ethidium bromide stained 5%PAGE, the middle and lower figure show autoradiographs of Southern blots of the amplification products probed with the IgHJ and bcl-2 probes respectively.

8 to 10) and JC (lanes 11 to 13) although no bands could be seen in patient JK (lanes 14 to 16). The band sizes are summarised in Table 2.4. The PCR products were resolved by agarose gel electrophoresis, Southern blotted and hybridised with JH and bcl-2 probes to confirm their origin in the translocated sequences. Therefore 4 of the initial group of 5 patients had a t(14;18) translocation with an mbr breakpoint. The patient without a demonstrable mbr translocation event did have tumour detectable by Southern blotting, therefore the negative result was not due to the integrity of the material under study or degree of tumour infiltration.

To confirm the origin of the 3 bands obtained when the different JH amplimers were used, the PCR products from patient SP were sequenced (Figure 2.13). All 3 sequences were identical, except at the 3' end, which varied depending on which amplimer was employed. The size difference of 23bp was due to the binding of the J6 amplimer 23bp downstream of the J1,2,4,5 and J3 amplimers. The presence of all three bands indicates the selection of IgHJ segment 6, as the binding site for J6 is not present on J segments 1 to 5. Sequence analysis was carried out on the remaining three patients to confirm this. The nucleotide sequence data from the junctional regions are shown in Table 2.5 indicating that J6 was involved.

Patient	Band Size (bp)
SP	191,208
LW	229,251
JC	282,301
JI	234,257

Table 2.4 Size (bp) of PCR amplification products of patients with low grade NHL amplified with bcl-2(25mer) and J1,2,4,5; J3; J6 primers.

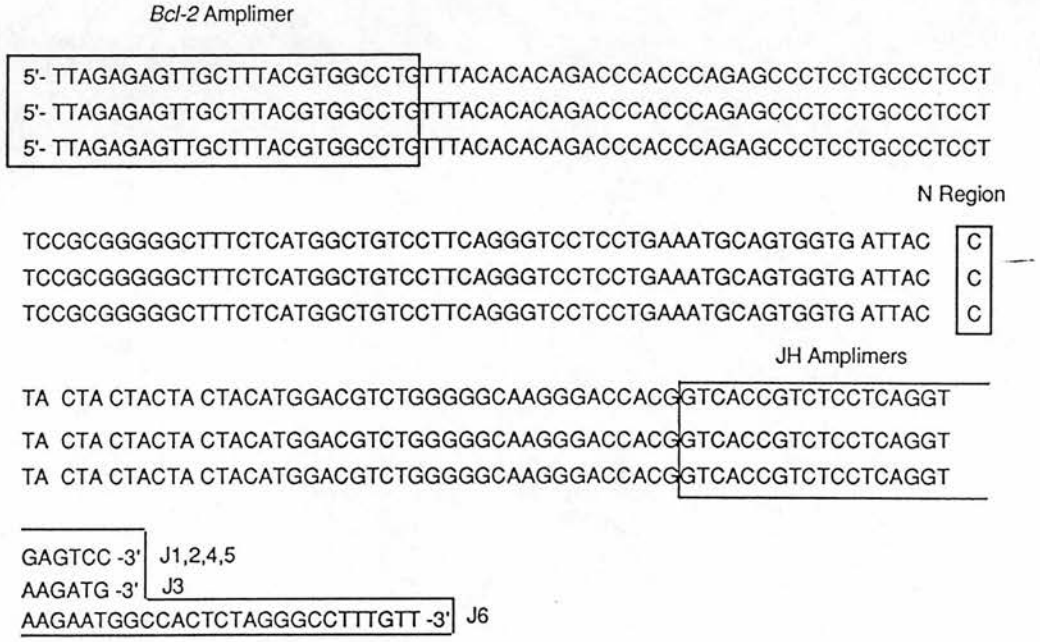


Figure 2.13 Sequence analysis of PCR products from a patient with a t(14;18) translocation. Each PCR product from separate amplifications using the 5' bcl-2 primer with individual 3' JH primers (J1,2,4,5; J3 and J6)(Table 2.1) was directly sequenced using the amplimers as sequencing primers. The bcl-2 and JH amplimers are boxed in the diagram with a single N nucleotide insertion also boxed.

Bcl-2	NDN	JH
AM 5'- CCGCGG 73	TAATGGGGTGT	CTTTGACTACTGGGGCCAGG-3' J4
IM 5'- CCCTCC 55	CTCTTTTCGTCATTTCGGTTGGGGCAGC	AACTGGTTCGACCCCTGGGGCC-3'J5
CM 5'- TGCTTA 125	ATTACCAGCAGGGAAA	TACGGTATGG-3' J6
WG 5'- TCCCCG 175	AGGTTAATC	GG 3'- J6
J C 5' - TCCCCG 175	CTGCGAGCGAGCCTACATCCTTTGACGGCCGTTGTGAGCTCGGTCCTCGCCAGAAAATC	TACTACTACTACGGTATGG 3'- J6
SP 5'- GCTTAC 126	C	TACTACTACTACATGGACG-3' J6
J I5 '- CCCCGG 174	CCTGGGTGATTAAC	ACTACTACTACGGTATGG3'- J6
LW 5' - CTCCCC 176	GAGGCTTGA	ACGGTATGG3'-J6

Table 2.5 Sequence analysis of PCR products from 8 patients (IM, SP, JI, JC LW, AM, CM and WG) with a t(14;18) translocation. DNA was amplified using the bcl-2 (20mer) and IgHJ1-6 consensus primers and the products directly sequenced.

2.5.3 PCR consensus amplimers

2.5.3.1 Sensitivity

The sensitivity of the second primer system using the bcl-2 (20mer) and JH consensus amplimers was tested as above. The PAGE gel of the amplified dilution products is shown in Figure 2.14 and the same products slot blotted and hybridised against the bcl-2 probe in Figure 2.15. Again sensitivity demonstrated by PAGE analysis is at the level of one tumour cell in 10000 HL60 cells, with a tenfold enhancement provided by slot blotting.

2.5.3.2 Screening

A total of 22 patients, on whom lymph node or involved marrow was available were screened using this PCR system. On amplification, marker bands were present in 8 patients (36%) after PAGE analysis (Figure 2.16) with the band sizes and cytogenetic data summarised in Table 2.6. The origin of the bands was confirmed by Southern blotting the products and hybridising against JH and bcl-2 probes (Figure 2.16).

The patients previously found to be positive with the original PCR system were also positive when screened with the consensus JH amplimer, one patient remained consistently negative. Of the 14 patients found to be mbr negative by PCR, 9 were previously shown to contain significant tumour populations by Southern blotting indicating that the samples studied were suitable substrates for analysis therefore the negative results

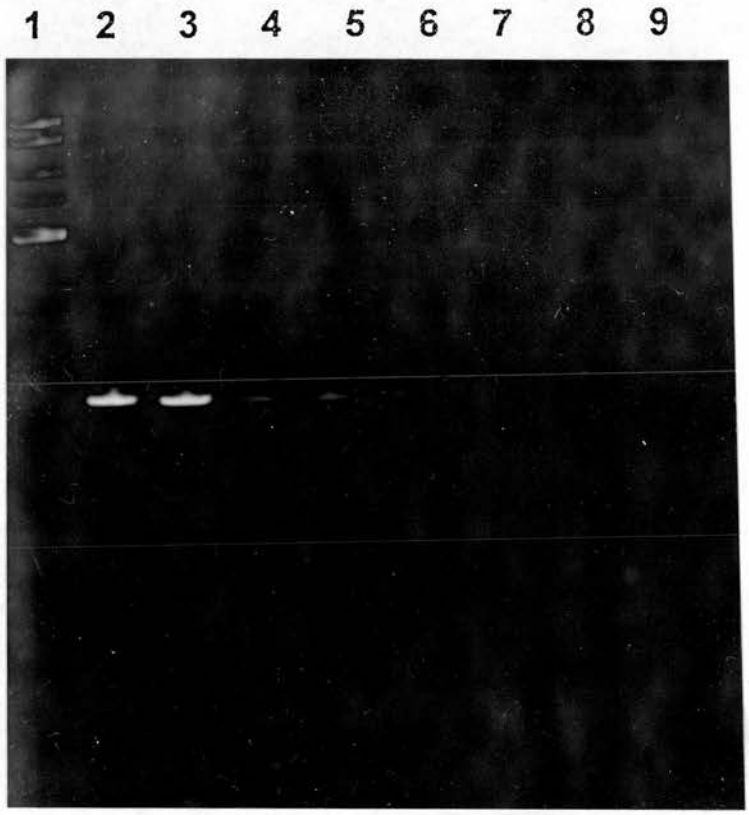


Figure 2.14 Ethidium bromide stained 5%PAGE of PCR amplification products of SUDHL6 DNA diluted in HL60 DNA. Lane 1 contains ϕX174 DNA digested with HinfI. Lanes 3 to 8 show tenfold dilutions of SUDHL6 amplified with the bcl-2 (20mer)$_1$ and IgHJ1-6 consensus primers. Dilutions range from 10^{-1} to 10^{-6} with undiluted SUDHL6 DNA in lane 2. Lane 9 contains no template DNA.

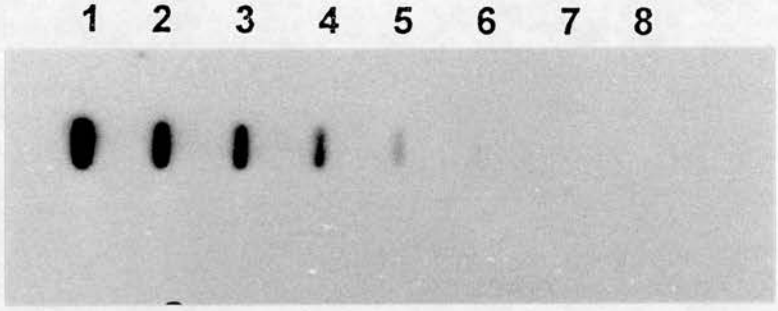
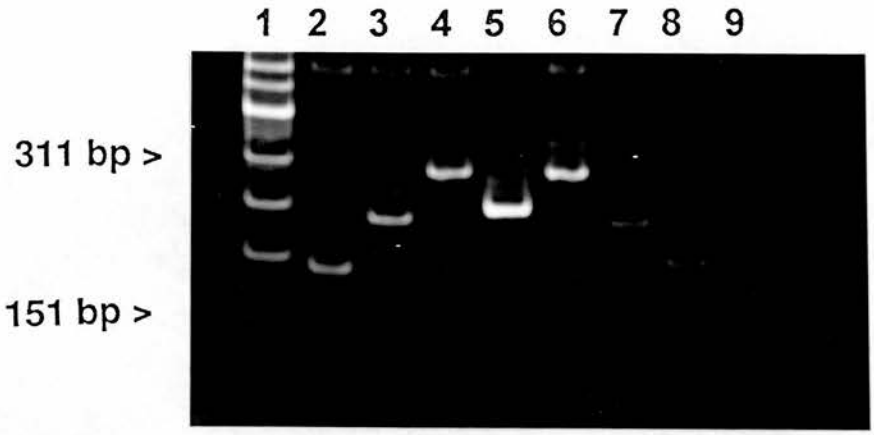


Figure 2.15 Autoradiograph of PCR amplification products of SUDHL6 DNA diluted in HL60 DNA and hybridised to the bcl-2 probe. Slots 2 to 8 show tenfold dilutions of SUDHL6 amplified with the bcl-2 (20mer)$_1$ and IgHJ1-6 consensus primers. Dilutions range from 10^{-1} to 10^{-6} with undiluted SUDHL6 DNA in lane 1. Lane 8 shows a negative control with no DNA template.



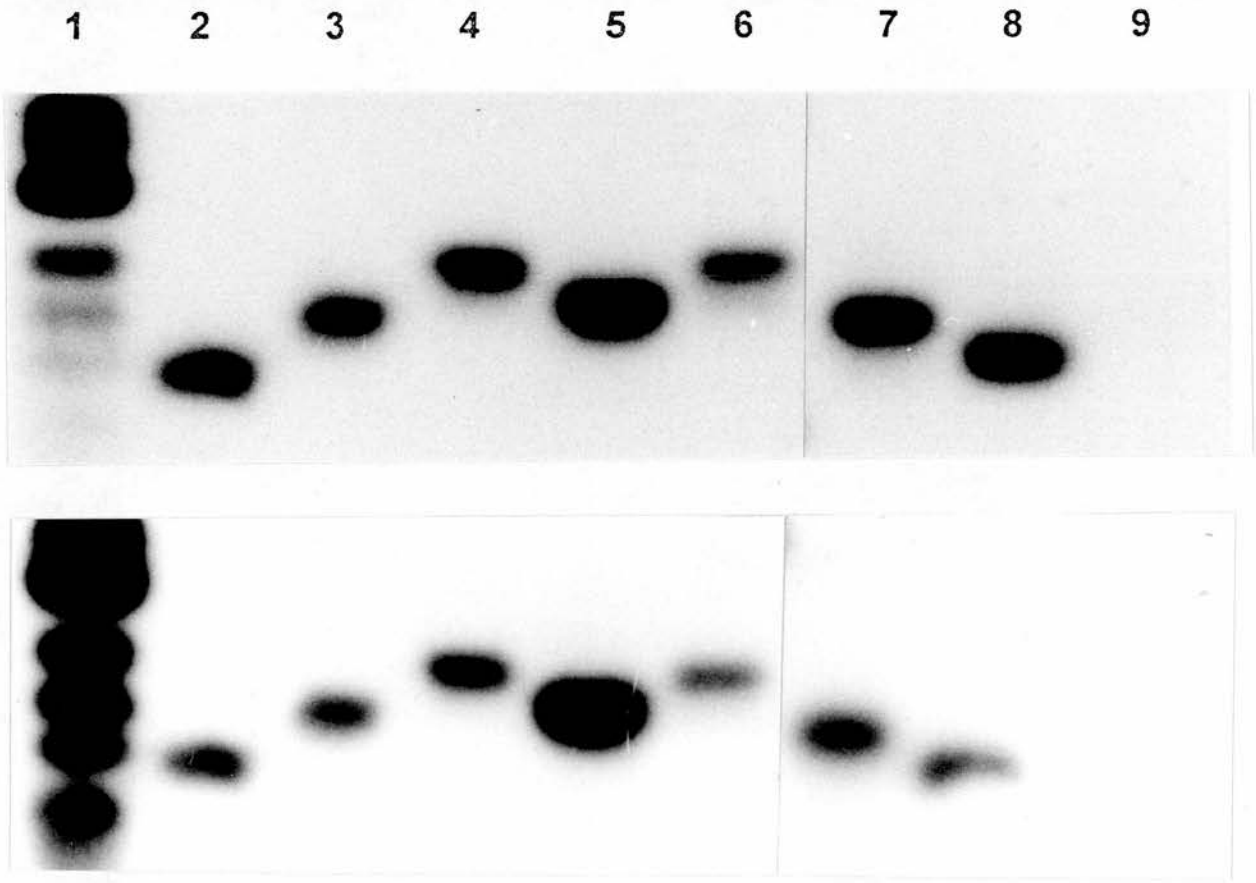


Figure 2.16 PCR amplification products of presentation lymph node DNA from 7 (lanes 2 to 8) patients amplified with the bcl-2 (20mer) and IgHJ1-6 consensus amplimers (Table 2.1). Lane 1 shows ϕX174 DNA digested with HinfI. Lane 9 contains normal DNA. The upper figure shows an ethidium bromide stained 5%PAGE, the middle and lower figure show autoradiographs of Southern blots of the amplification products probed with the IgHJ and bcl-2 probes respectively.

Patient	Southern	Cytogenetics	t(14;18)
SP	RR	14;18	195
LW	RR	14;18	234
JC	RR	14;18	295
JI	RR	14;18	234
IT	GR	14;18	-
WGr	GR	14;18	219
EB	RR	14;18	-
JS	GR	14;18	-
JK	GR	14;18	-
CL	RR	8;14	-
AMc	RR	8;14	-
HG	GR	NA	-
WGl	GR	14;18	-
IM	GR	NA	141
AMa	GR	NA	126
GN	RR	NA	-
AS	RR	ND	-
EW	RR	NA	-
JW	RR	NA	-
CM	ND	14;18	188
JB	ND	Complex	-
ML	ND	NA	-

Table 2.6 Summary of Southern blot, cytogenetic and PCR results from patients with low grade NHL amplified with bcl-2(20mer) and consensus JH primers. Band sizes are indicated in bp.

were not due to lack of tumour infiltration. Of the 10 patients known to bear t(14;18) translocations by cytogenetic analysis, 6 showed mbr PCR positivity suggesting that the remainder have mcr translocations or more distal mbr breakpoints.

Following PCR amplification with the consensus primer system and PAGE analysis, mbr translocations were not detected in any of the 8 patients with HD at the 10^{-4} level or above.

2.5.4 Sequence analysis

All 8 PCR positive patients were sequenced in both directions, using the PCR amplimers as sequencing primers, in order to assess JH usage and to map the breakpoints within the bcl-2 gene. A typical autoradiograph from a denaturing PAGE gel of such a reaction is shown in Figure 2.17 and shows chain extension from the bcl-2 and JH primers, with the junctional sequence indicated. Sequence data obtained on all 8 patients is shown in Table 2.5. Due to variation in the bcl-2 mbr breakpoints over a 150bp region, there is large variation in the amount of bcl-2 sequence involved in the translocation, 111bp separates patients IM and LW. The mbr breakpoint conversely varies by only 3 nucleotides between 4 patients (LW, JC, WG and JI), with 2 (JC and WG) occurring at the same base. No evidence for the involvement of D segments was found therefore the junctional nucleotides were classified as N nucleotides

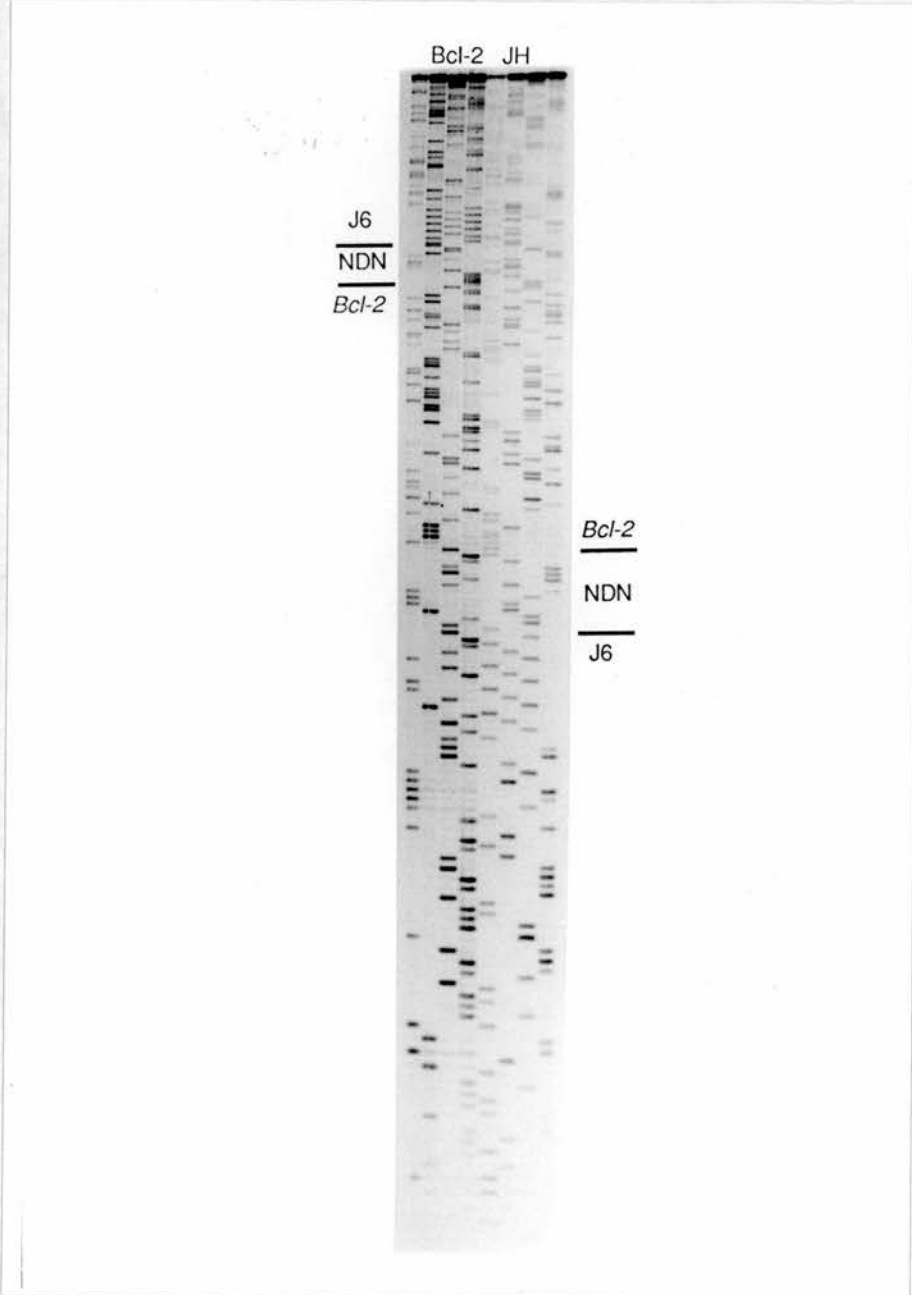


Figure 2.17 Autoradiograph of an 6% denaturing PAGE gel for sequencing PCR products from a patient with a t(14;18) translocation. Products were sequenced from the bcl-2 (20mer) (left) and IgHJ1-6 (right) consensus primers. Termination reactions were loaded in the order G, A, T and C.

which showed a GC content of 50%. Insertions varied from one (SP) to 59 (JC) nucleotides. Differing JH selection was also observed with 6 patients displaying J6, one patient (AM) using J4 and one (IM) using J5. Variable degrees of exonucleolytic "trimming" was observed, with only two patients showing intact J boundaries (SP and IM) with up to 21 nucleotides being deleted (WG). Two patients displaying J6 usage had a GGT triplet deleted from the germline sequence. A summary of the bcl-2 breakpoints described here in addition to those previously reported is presented in Figure 2.18.

In summary, clonal IgH rearrangements were detected in all 19 patients studied. PCR analysis generated specific products in 8 of 22 cases (36%), the nature of which were confirmed by sequence analysis which demonstrated tight clustering of breakpoints within a 100bp region.

2.6 DISCUSSION

Southern blotting with the IgHJ probe identified clonal expansions of lymphocytes in tumour DNA from all 19 patients with low grade lymphoma, however no such proliferations could be detected in 8 patients with HD. The latter finding is in accord with the published incidence of IgH rearrangements in HD, reported in approximately 10% of patients (Daus et al 1989).

PCR based techniques provide more sensitive methods for the detection of tumour cells, although care must be

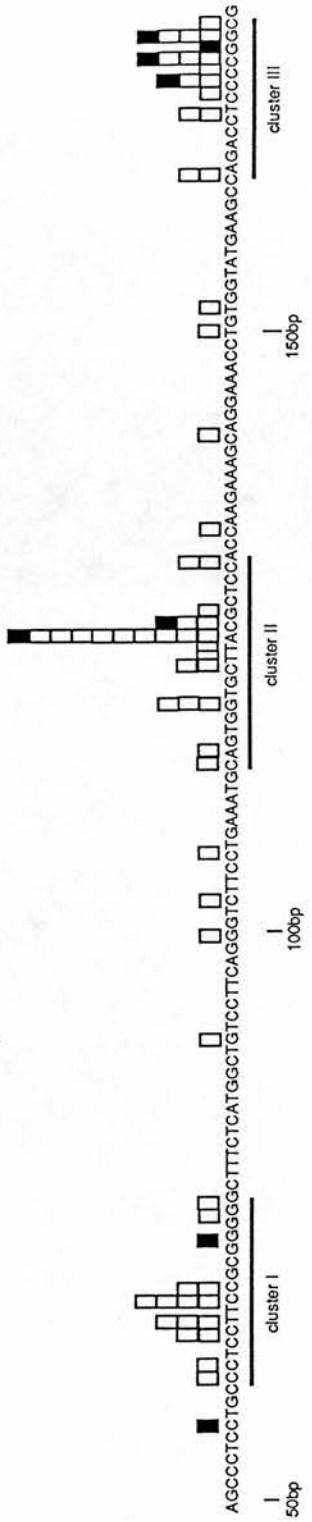


Figure 2.18 Map of nucleotides 50 to 177 of the mbr region of the bcl-2 gene. Open squares indicate breakpoints previously published (reviewed Wyatt et al 1992). Solid squares indicate breakpoints identified in our series of patients. Three breakpoint clusters are indicated by underlining.

taken in determining the optimal reaction conditions in order to ensure consistency and simplify the interpretation of results. Specific amplification was improved by the careful selection of primer sequences and by determination of optimum magnesium ion concentration. Excessive numbers of cycles were found to increase non-specific banding without an increase in yield. The amenability of PCR to the analysis archival material, such as frozen sections, increased the number of patients available for analysis to 22. Previous studies have reported the incidence of mbr breakpoints in 30 to 70% of low grade NHL patients with a t(14;18) (Amakawa et al 1989, Weiss et al 1987) and the overall frequency of mbr translocations in this study, regardless of cytogenetic status, was 36%. Among patients previously characterised as bearing t(14;18) translocations, 6 out of 10 produced a discrete amplification product. Pezzella et al (1990) reviewed the overall incidence of mbr and mcr translocations and reported these to be 35.3% and 5.9% respectively. According to these figures, an mcr translocation would occur in approximately 1 of 22 patients in this study, although this was not investigated.

The initial primer system used (Stetler-Stevenson et al 1988) employed 3 JH specific primers, however due to lack of specificity to the particular JH segments to which they were designed, a single consensus JH primer was designed. This contains a single bp mismatch with J3, but

this did not lead to an under representation of this sequence when used for CDRIII amplification (discussed in Chapter IV). Dilutions of the t(14;18) SU DH L6 line in a myeloid cell line demonstrated the sensitivity of the technique, which was able to detect a single copy of a translocated sequence in a background of 10^5 normal sequences. PAGE analysis of the amplification products of only 10 cells yielded a clear signal, which has implications for the study of archival or specimens on which little material is available.

Sequence analysis using a simple direct sequencing technique which avoided asymmetric PCR or sub-cloning was confirmed the source of the bands demonstrated by electrophoresis. Some of the features of normal IgH V-D-J recombination events (Tonegawa 1983), such as N insertion and exonucleolytic trimming of JH segments was evident. Other groups have reported the involvement of D segments (Eick et al 1990, Cotter et al 1990, Kneba et al 1991) however no evidence for this was found in this series. Clustering of mbr breakpoints was evident with 111bp separating the most distal, but it is possible that breaks 5' of the bcl-2 primer could occur. Wyatt et al (1992) characterised one breakpoint one kb 3' of the region designated cluster 3 but any occurring more 3' of this may not amplify to be detected. Breakpoints outwith the mbr are relatively rare but those proximal can be detected by Southern analysis (Pezzella et al 1990) or those more distal by PFGE (Zelenetz et al 1991). The

application of FISH with chromosome 14 and 18 probes to interphase cell preparations would also be effective in the detection of translocation events, however this does not permit the study of preserved material and the sensitivity would be less than that of PCR.

Variation was seen by with the insertion of differing lengths of N nucleotides though a high GC bias was not apparent as in other immune gene junctions. This was also observed by Kneba et al (1991). A bias towards J6 selection was also clear with varying exonucleolytic trimming apparent at all JH termini. One patient had a GGT triplet deleted 15 nucleotides from the 5' boundary of J6. The N-J junctional sequences therefore resembles the junctional regions produced by regular IgH recombination events although more extensive N nucleotide insertion was present in some cases. The signal sequences which would mediate the bcl-2-(D)-JH recombination have not been clearly demonstrated. Wyatt et al (1992) first characterised the clustering of bcl-2 breakpoints in 3 distinct regions within a 150bp region of the mbr. Each cluster region was associated with an 8 nucleotide sequence homologous to the prokaryotic chi recombination initiation signal which they suggested mediated an initial bcl-2-DXP recombination. This was then followed by a DXP-JH event before resolution by the regular IgH recombinase mechanism. They also observed duplication of mbr sequences and the presence of DXP signal sequences in some junctions but these were not observed in the 8

patients sequenced here. All the bcl-2 breakpoints studied fell within the 3 cluster regions described by Wyatt, with one exception, which was located 4bp 5' of cluster region 1. Interestingly a tight grouping of 4 breakpoints was observed in cluster region 3. Further analysis of translocation signal and coding sequences will be required in order to understand the mechanisms underlying non-homologous recombination.

Eick et al 1990 exploited sequence data to generate clone specific oligonucleotides to monitor disease, in order to minimise the occurrence of false positives. Kneba et al (1991) also designed clone specific oligonucleotides but used these as primers for a clone specific PCR amplification following an initial amplification with regular bcl-2 and consensus JH primers. These techniques do not provide an enhancement in sensitivity but do ensure specificity and enable non-radioactive detection of rare species however as different oligonucleotides do not amplify uniformly and require varying reaction conditions, this approach may be limited in certain patients. Price et al (1991a) used "booster" and nested PCR in order to generate sufficient material to allow sequence analysis of very rare t(14;18) species from DNA samples obtained in remission. Nested PCR reactions can lead to carry over problems as separate equipment, reagents and preparation areas are required for each subsequent stage. The single step system described here is straightforward and sufficiently sensitive to detect a

single translocated sequence and is potentially less prone to carry over than nested systems although it does not facilitate the characterisation of rare species generated in the course of therapy. However as these species did not replace the initial clone at relapse, this is not a necessary step in order to successfully follow tumour progression. Also, these rare species may be derived from translocation events occurring in normal lymphocytes, as detected by Limpens et al (1992) in enriched B-cell populations. This may explain the finding by Stetler-Stevenson et al (1990) of t(14;18) events in lymph nodes from patients with HD. These translocations may have originated in reactive lymphocytes rather than Reed-Sternberg cells.

In conclusion, t(14;18) translocations were detected in 36% of patients with low grade NHL, but not detected in 8 patients with HD. Optimisation of reaction conditions provides a sensitive disease marker for the determination of MRD in a proportion of low grade NHL patients undergoing therapy. Sequence analysis confirmed the origin of the markers and demonstrated features associated with normal immune gene junctions and clustering of mbr bcl-2 breakpoints. If residual disease analysis is to provide meaningful results, carry-over must be avoided by careful laboratory technique and monitored by the rigorous use of control reactions.

CHAPTER III

T-CELL RECEPTOR δ CHAIN IMMUNOGENOTYPE ANALYSIS

3.1 INTRODUCTION

3.1.1 T-cell receptor αβ complex

T-lymphocytes are conditioned in the thymus to recognise foreign antigens presented in a "self" MHC context mediated via a transmembrane glycoprotein heterodimer non-covalently linked to the signal transducing CD3 complex on the cell surface (Saito et al 1987). The most prevalent heterodimer is the TcR αβ complex, found on the majority of peripheral T-cells. It is associated with either a CD4+, CD8- or CD4-, CD8+ single positive (SP) phenotype corresponding to helper and killer cells respectively (Lanier and Weiss 1986). A signal transduction cascade is activated by the interaction of the TcR complex with antigen which results in cell proliferation and secretion of IL- 2 by mature T-cells.

The generation of immune diversity is mediated by a recombinase system analogous to that involved in Ig gene rearrangements whereby germline encoded segments are recombined to produce a unique TcR gene (Saito et al 1984). This involves endonucleolytic cleavage and ligation of random germline segments mediated by heptamer/ nonamer RSS and a recombinase enzyme. This

generates a chromosomal coding joint and a closed circular extrachromosomal signal joint. A polyclonal population of T-cells will therefore bear the diverse repertoire of TcR genes necessary for antigen recognition. The TcRα locus has >50 variable (V) segments and approximately 55 joining (J) segments and the β locus >70 V segments and 13 J segments as well as 2 diversity (D) segments, providing extensive combinatorial diversity (Davis and Bjorkman 1988). Further variability is provided by random exonucleolytic digestion of these germline encoded segments and insertion of non-template encoded N nucleotides at the junctions mediated by the enzyme TdT. During development in the thymus, T-cells undergo positive selection for the ability to recognise foreign antigens in the correct MHC context and negative selection to remove autoreactive TcR's. These steps are regulated by the $\alpha\beta$ complex in addition to its role in T-cell activation (Finkel, Kubo and Cambier 1991).

3.1.2 Characterisation and structure of the T-cell receptor δ- chain locus

After the predominant CD3 associated TcR complex was characterised, a population of CD4-, CD8- thymic T-cells expressing CD3 in conjunction with a novel protein, designated TcR γ (Saito et al 1984), was described (Brenner et al 1986). A combination of FACS analysis and immunoprecipitation with framework region monoclonal antibodies followed by Northern blotting confirmed the existence of this T-cell subset that lacked functional α

or β chain expression. Two molecules of molecular weight 55kD and 40kD co-precipitated with the CD3 complex from these cells, with the 55kD molecule corresponding to the inferred weight of the fully glycosylated TcR gamma protein. The second molecule was therefore designated the TcRδ chain, which was not initially found to be disulphide linked to the γ chain.

The TcRδ locus was mapped to chromosome 14 and localised within the TcRα locus (Chien et al 1987), nested between the Vα and Jα segments. The Cδ locus mapped 85 kb upstream of the Cα region and these genes were found to be homologous (Takihara et al 1988) indicating a possible duplication event in the divergence of the TcR loci. Three Jδ and 2 Dδ segments were sequenced, displaying recombination signal heptamer and nonamer sequences consistent with those previously characterised in other immune receptor genes. Further studies identified 6 V, 3 D and 3 J segments, hence the amount of germline encoded diversity was extremely small compared to that of the α and β loci (Takihara et al 1989). Sequence analysis of rearranged TcRδ genes from thymic cell lines showed a further restriction of germline variability due to the restricted selection of V and J segments (Loh et al 1988). Frequent selection of Vδ1 and Jδ1 was apparent, with the rarer selection of a second Vδ segment but the actual junctional diversity was found to be enormous due to the selection of multiple Dδ segments that could be read in all 3 frames, extensive exonucleolytic trimming

of germline sequences and the insertion of non-germline encoded N nucleotides mediated by the enzyme TdT (Hata et al 1988). Single sided or "anchored" PCR (A-PCR) of mRNA which enabled amplification with a single specific primer facilitated the characterisation of previously unknown V segments recombined to Jδ1 segments expressed in peripheral T- cells (Loh et al 1989). All the sequences obtained were distinct and in frame, and again restricted V selection was apparent, with only 2 members of the Vδ family observed, Vδ1 and Vδ2. This indicated a difference in the repertoire of V selection between cells of thymic (selecting primarily Vδ1 and Vδ3) and peripheral T-cell origin (selecting Vδ1 and Vδ2). Further to this, the previously described multiple Dδ segment selection observed in thymic cells was less frequent. The selection of Vδ segments proximal to the Dδ/Jδ1 region appears to explain such preferential selection (Greisinger et al 1991). This may be mediated by the inaccessibility of more distal segments to a recombinase enzyme bound at a particular site perhaps due to the local chromatin structure (Tycko, Coyle and Sklar 1991).

The localisation of the TcRδ locus within the TcRα locus indicated that the TcRδ chain would rearrange at an early stage as recombination of the TcRα locus would remove the TcRδ genes. Signal sequences flanking the TcRδ locus mediating its deletion have been determined, the 5' δrec and 3' pseudo Jα regions (De Villartay et al 1988). When the TcRδ locus was mapped to the TcRα locus, it was

assumed that V segments could be shared between the 2 chains. However, the V segments characterised were distinct although some Vδ segments are interspersed within the Jα cluster and some RSS are shared. Chimeric TcRαδ rearrangements have been reported but these arise via a mechanism that bypasses the δrec signals and does not involve the shared signal sequences. This results in a VδDδ-Jα molecule which may be an intermediate of a mature TcR Vα-Jα molecule (Yokota, Bartram and Hansen-Hagge 1991b).

Another feature of TcR and other immune gene junctions is the presence of P or palindromic nucleotides, previously assumed to be TdT mediated N insertion but may actually be germline encoded (Lafaille et al 1989). These nucleotides are found at the intact boundaries of germline segments, are of one to 2 nucleotides in length and are palindromic with respect to the adjacent germline sequence. They were postulated to arise during the recombination process via the "nicking" of one strand 2 base pairs from the boundary of the segment selected, followed by inversion of the 2 terminal nucleotides and repair of the newly created 4 nucleotide single stranded projection to generate a short palindrome. Exonucleolytic digestion at the junction would remove one or more nucleotides, hence these P nucleotides occur only at intact germline segments.

Restricted combinatorial germline diversity has also been

reported at the TcRγ locus on chromosome 7. Fifteen V segments were described, of which 8 are functional along with 5 J segments and 2 C regions, the selection of which determines the disulphide linkage status of the γ/δ complex. Mature TcR heterodimers involving C γ1 are disulphide linked whereas those involving C γ2 are not due to the lack of a cysteine residue in the second exon (Krangel et al 1987). Although TcRγ bearing lymphocytes are rare, they are abundant in epithelial tissues (Itohara et al 1990a) with subgroups of these lymphocytes expressing TcR's which are highly homogeneous, both at the junctional and combinatorial level (Lafaille et al 1989).

3.1.3 T-cell ontogeny

The precursors of T-lymphocytes are thought to originate in the bone marrow before migrating to the thymus (Ezine, Weissman and Rouse 1984) where they develop, via an ordered process involving TcR gene rearrangement to mature thymocytes. Positive selection for MHC context and negative selection for autoreactivity was identified for TcR$\alpha\beta$ T-cells (Davis and Bjorkman 1988), but although selection is involved in $\gamma\delta$ T-cell development, an extra thymic component may be involved (Dent et al 1990).

High levels of TdT is expressed in the nuclei of immature lymphoid cells (Bollum 1979) suggesting the onset of immune gene rearrangement, with persisting expression in cortical thymocytes, but expression is not found in

mature T-cells (Foon and Todd 1986).

Studies of T-ALL suggest that it represents a clonal expansion of cells arrested at different stages of T-lymphocyte development in different patients, resulting in a range of TcR gene rearrangements. Such cells are mainly CD3 negative and do not express mature surface TcR molecules. Many have both β and γ chain gene rearrangements, though some show γ chains only, suggesting that γ genes rearrange before β whereas α-chain involvement is rare indicating a later rearrangement (Felix et al 1987). The majority of T-ALL have one or 2 δ-chains rearranged or deleted, with the gamma and β chain genes in germline configuration in some cases (Foroni et al 1989) hence δ-chain rearrangement would appear to occur first.

This pattern of rearrangements suggests an ordered hierarchy of TcR gene rearrangements and some of the regulatory elements mediating this process have been described. An unusual 2.0kb TcRα mRNA species lacking a Vα region was found in early T-cells and was designated T-earlyα (TEA-Cα) (Hockett et al 1988). The gene coding for this transcript is in the germline configuration in immature T-cells and TcR $\gamma\delta$ lymphocytes but deleted in TcR$\alpha\beta$ T-cells. Such transcription may have a role in rendering the Jα locus accessible for δ-chain gene deletion via the δrec/ pseudo Jα mechanism thus enabling α-chain rearrangement.

Rearrangement events result in the excision of circular extrachromosomal elements bearing the signal sequences and these can be studied and intermediate structures analysed (Fujimoto and Yamagishi 1987). Such studies have indicated a progressive mechanism whereby germline or non-functional δ-chain rearrangements can be deleted, followed by α-chain rearrangements which in turn can further recombine to form new genes using upstream Vα and downstream Jα segments (Takeshita et al 1989).

Tissue specific enhancer elements are involved in the transcriptional activation of the TcR locus which are silenced in non T-cells by cis acting "silencer" elements (Winoto and Baltimore 1989). Two δ-chain enhancers located between Jδ3 and Cδ have been described which are T-cell specific and active in both γδ and αβ lymphocytes. A cis-acting γ gene silencer which prevents γ chain transcription in mice has also been reported (Ishida et al 1990, Bonneville et al 1990).

Van Dongen et al (1990) proposed a coordinated model for TcR gene rearrangement during T-lymphocyte maturation involving a pivotal role for the TcRα/δ locus. The earliest event is the activation of TcRδ enhancers resulting in TcRδ rearrangement followed by concomitant γ and β-chain rearrangements. Ultimately γδ T-cells would result if activity of the α-silencer

persisted repressing α-chain rearrangement. Inactivation of the α-silencer would lead to transcription mediated opening of the α locus via TEA-Cα and exposure of the pseudo Jα element. The δrec element would already be accessible due to previous δ-chain gene activation, leading to α-chain gene recombination and the subsequent production of αβ T-cells.

The process of thymic selection of αβ T-cells for restricted MHC recognition is thought to be mediated by the CD4 and CD8 molecules, however few peripheral γδ T-cells bear either molecule. This suggests that γδ cells may mature via an extra thymic pathway, supported by the fact that few TdT positive γδ thymocytes have been observed, most being TcRαβ although the junctional regions of the former are far more diverse. A study of children with Di George's anomaly (DGA) in which varying degrees of thymic aplasia are found enabled the relationship of thymic mass and T-cell repertoire to be studied (van Dongen et al 1990). In healthy children mature T-lymphocytes are mainly TcRαβ positive whereas in DGA the levels of TcRαβ are affected by reduction in thymic mass while γδ cells remain virtually constant.

Further to this, the number of peripheral T-cells in healthy children falls until adolescence, mainly affecting αβ lymphocytes as the thymus involutes which agrees closely with the relationship of thymic mass and the level of αβ T-cells in DGA patients. These findings

indicate that at least part of the maturation process of $\gamma\delta$ T-lymphocytes must occur at some as yet uncharacterised extra-thymic location.

3.1.4 Involvement of the TcRα/δ locus in leukaemia

The analysis of various leukaemias by Southern blotting with TcR probes has demonstrated the frequent association of δ-chain rearrangements with different leukaemia sub-types (Felix and Poplack 1990). These can involve complete or incomplete V(D)DJ rearrangements in addition to translocations recombining the α/δ locus to other loci including oncogenes which may have a direct role in tumour aetiology.

Translocations associated with T-lineage ALL usually involve the α/δ locus as opposed to either β or gamma genes, perhaps due to the early rearrangements of the δ locus in lymphocyte ontogeny (Rabbitts and Mengle-Gaw 1988). Translocations involving known oncogenes such as the t(8;14)(q24;q11) which juxtapose the α/δ locus on 14q11 next to c-myc on 8q24 probably have a direct role in tumour aetiology. Some sporadic translocations, such as inversions involving the α/δ and IgH loci are probably not related to the tumour lesion and represent a rare event in cells which have been rescued by a progression to neoplasia.

Recombination of the Tal-1 locus on 1q34 to the α/δ locus is found in 3% of T-ALL although site specific deletions

of the Tal-1 gene are associated with a further 26% (Brown et al 1990, Chen 1990). This gene encodes a helix-loop-helix protein and such genes have a DNA binding affinity these are involved in developmental gene regulation hence such a lesion probably has a direct role in tumourigenesis in addition to being a useful marker of disease.

Rearrangements of TcR genes are found in all cases of $\alpha\beta$ and gammaδ expressing T-ALL's as described above but in addition, frequent rearrangements of β, γ and δ genes were reported in B-lineage ALL (Felix et al 1990). Restricted repertoires of TcRδ gene selection were reported in both T- and B-ALL with the majority of T-ALL selecting Vδ1 or Vδ2 with Jδ1. Complete V-D-J δ-chain rearrangements were not common in B lineage ALL but deletions at the TcRδ locus are frequent and recombinations involving Vδ2 and Dδ3 have been observed in approximately 50% of patients (Biondi et al et al 1990). Further rearrangements to Jα genes creating chimeric Vδ2Dδ3Jα molecules have been reported in 27% of cases of B-lineage ALL (Yokota, Hansen-Hagge and Bartram 1991b).

3.1.5 Analysis of minimal residual disease

The enormous diversity at TcR junctions has the potential to provide extremely specific markers for clonal populations of neoplastic cells. The restricted repertoire of germline flanking sequences associated with

disease subtypes are suitable for the selection of PCR amplimers. In addition, the relatively low reported incidence of oligoclonality and clonal evolution at this locus may provide a stable marker for disease (Beishuizen et al 1992).

Vδ1-Jδ1 rearrangements found in 25% of T-ALL were the first TcR genes exploited as a tumour specific marker (Hansen-Hagge, Yokota and Bartram 1989). A "clonospecific" probe was generated by amplifying the junctional sequence from tumour material at presentation which appeared as a discrete band on resolution by agarose gel electrophoresis. After therapy, the diminished level of neoplastic cells would result in the amplification of a polyclonal population and the tumour band would be masked by sequences from normal lymphocytes. The presence of neoplastic cells within a background of normal cells could then be discerned by hybridisation with the "clonospecific" probe. Dilution of tumour cells in normal mononuclear cells demonstrated the sensitivity of this technique at the 10^{-6} level i.e. if the equivalent of 1000000 cells (10μg of DNA) was amplified, then one tumour cell could be discerned.

Neale et al (1991) extended this approach by designing primers to Vδ1, Vδ2 and Vδ3 and Jδ1, Jδ2 and Jδ3 which they used to screen T-ALL patients in pairwise configurations. This detected TcR rearrangements in 76% of patients, the majority being Vδ1-Jδ1 as expected. They

sequenced the PCR product and used this information to design anti-junctional oligonucleotide probes to monitor remission populations of neoplastic cells. Dilution experiments estimated the sensitivity of the system to be at the level of one tumour cell in a background of 100 000 normal cells if 1μg of DNA was examined.

These approaches were restricted to a subgroup of T-lineage ALL patients to be analysed, but were extended by exploiting the Vδ2-Dδ3 partial rearrangement frequently found in B-lineage ALL (Yokota et al 1991a). However, this is only a partial rearrangement and provides less diversity. Probes raised against this junction may be less sensitive in some cases than those against the Vδ1-Jδ1 junction but can still be successfully applied to detect residual disease.

3.1.6 Aims

I wished to determine the incidence of TcRδ-chain rearrangements in a range of haematological disorders and investigate the potential application of PCR analysis to the detection of residual disease. Initially patients were screened by Southern blotting before the development of a PCR system to provide rapid screening and sensitive disease detection. Sequence analysis was performed to confirm the nature of the amplification products and evaluate to the degree of diversity shown at this locus and therefore the effectiveness of clone specific probes in studying disease in patients undergoing therapy.

3.2 PATIENTS

3.2.1 T-cell ALL

A group of 8 patients with T-cell ALL (3 male, 5 female) with a mean age of 27 years (range 5-71 years) were studied.

3.2.2 B-lineage ALL

A total of 14 adults (8 male, 6 female) with a mean age of 33 years (range 16-60 years) were available for analysis along with 22 children (10 male, 12 female) with a mean age of 6 years (range 2-14 years).

3.2.3 T-cell NHL

Ten patients (7 male, 3 female) with a mean age of 60 years (range 20 to 74) were screened.

3.2.4 AML

Sixteen patients with AML (9 male, 7 female) with an average age of 41 years (range 30 to 75) were studied.

3.3 MATERIALS

The materials were as detailed in section 2.3 with the addition of the following reagents.

TMACl wash solution

TMACl	3.0M
Tris pH8.0	0.5M
EDTA pH8.0	0.002M
SDS	0.1%

T4 Polynucleotide kinase (Amersham)

dATP gamma ^{32}PdATP (DuPont)

TMACl (Camlabs, Cambridge, U.K.)

3.4 METHODS

The methods were as detailed in section 2.4 with the following additions.

3.4.1 Restriction endonuclease digestion and Southern blotting

This was performed as section 2.4.2 except genomic DNA was digested with HindIII or BglII.

3.4.2 Probes

TcRδ chain gene rearrangements involving Jδ1 were detected with a 1.2kb Jδ1 SacI fragment designated JδS16 (Boehm et al 1988) and is illustrated in Figure 3.1.

3.4.3 PCR analysis of the Vδ1-Jδ1 rearrangement

This involved an approach essentially as described by

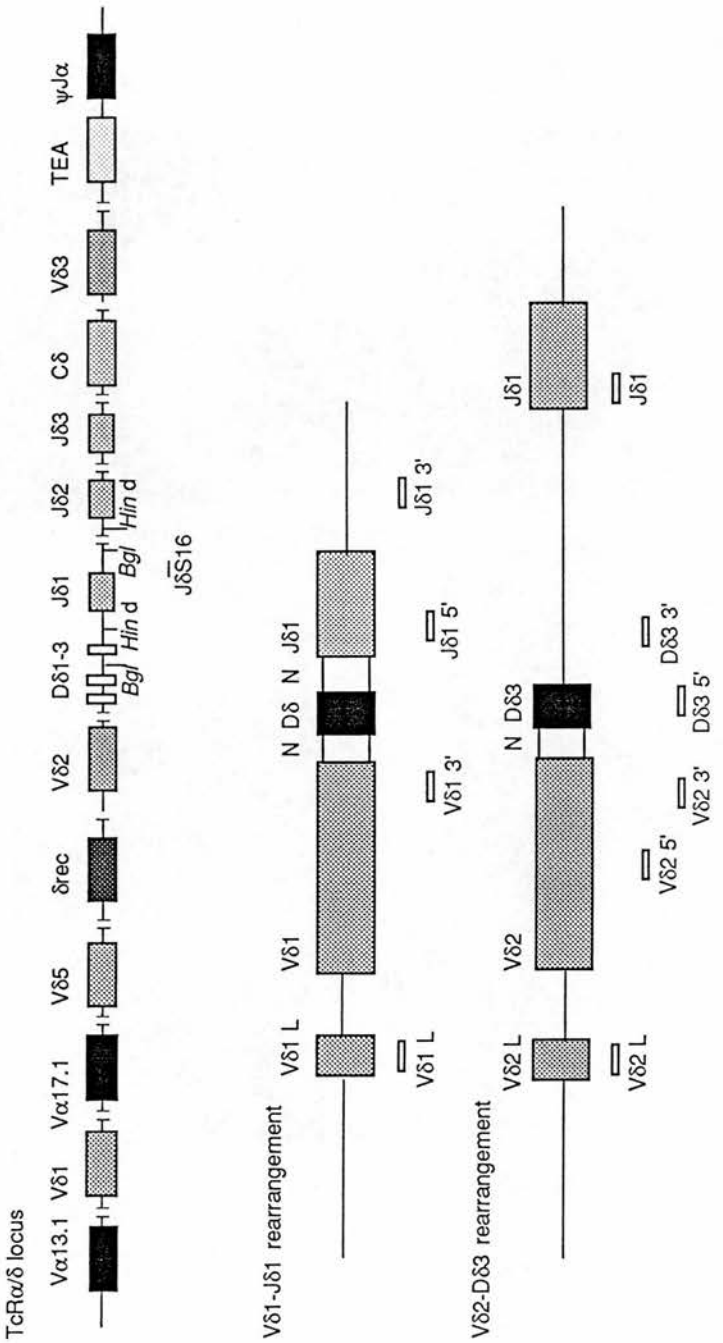


Figure 3.1 Diagram of the TcRδ locus. The germline configuration of the TcRδ gene segments is shown in the upper diagram with the location of the Jδs16 probe, HindIII and BglII restriction enzyme recognition sites indicated. The middle shows a Vδ1-D-Dδ1 recombination with the amplimer binding sites indicated by boxes. The lower diagram shows a Vδ2-D-Dδ3 partial recombination event with the amplimer regions indicated.

Hansen-Hagge et al (1989) which exploited the conserved Vδ1-Jδ1 rearrangement associated with T-cell ALL. An initial set of PCR cycles was performed using outer Vδ1 L (leader) and 3'-Jδ1 amplimers followed by a second nested step using 3'-Vδ1 and 5'-Jδ1 amplimers (Table 3.1). The product of the second cycle was excised from an agarose gel, digested with a restriction enzyme to remove 3' germline sequences, radiolabelled and used as a "clonospecific" probe to monitor disease progression in patients undergoing therapy.

3.4.3.1 Polymerase chain reaction analysis

Each reaction contained 1μg DNA, 1x PCR buffer, 200μM each dNTP, 30pmol each primer, 1U Taq polymerase and 0.01% gelatin. Cycles were as detailed in Table 3.2. The first round of amplification was performed with outer primers Vδ1 L (leader) and Jδ1 3' (Table 3.1). An aliquot of this first reaction (1%) was transferred to a second reaction with fresh reagents and amplification performed with primers Vδ1 3' and Jδ1 5'. A magnesium titration was performed using presentation tumour DNA from a T-ALL patient with a biallelic Vδ1-Jδ1 rearrangement as template (Figure 3.2). Lane 2 contains the first round product (approximately 700bp) and lanes 3-6 the second round product amplified with 1-2.5 mM $MgCl_2$ in 0.5 mM increments. For both primer pairs, the optimal value was 2mM $MgCl_2$.

Vδ1-Jδ1

Vδ1 L	5'-GTGTGTATTTGTGGCCTTCA-3'
Jδ1 3'	5'-AAATGCTAGCTATTTCACCCA-3'
Vδ1 3'	5'-GCAAAGTACTTTTGTGCTCTTG-3'
Jδ1 5'	5'-GGGTTCCTTTTCCAA**G**GATGAG-3'

labelling hexamer 5'-CTCTTG-3'

Vδ2-Dδ3

Vδ2 L	5'-TCATCCATCTCTCTCTCTTC-3'
Vδ2 5'	5'-GAGTCATGTCAGCCATTGAG-3'
Vδ2 3'	5'-GCACCATCAGAGAGAGATGA-3'
Dδ3 5'	5'-AGGGAAATGGCACTTTTGCC-3'
Dδ3 3'	5'-TTGTAGCACTGTGCGTATCC-3'

labelling hexamer 5'-GTGCCT-3'

Table 3.1 Nucleotide sequence of PCR primers for amplification of the Vδ1-Jδ1 and Vδ2-Dδ3 loci. The artificial FokI site is underlined within the Jδ1 5' sequence, the altered nucleotide is in bold type. Hexanucleotide labelling primer sequences are also shown.

Temperature (C)	Time (s)	Number Of Cycles
92	720	
56	120	1
add enzyme		
72	90	
92	30	
56	60	35
72	90	

Table 3.2 Cycling conditions for Vδ1-Jδ1 and Vδ2-Dδ3 amplification. For the inner (nested) reaction the initial set of cycles with the elongated denaturation step is omitted.

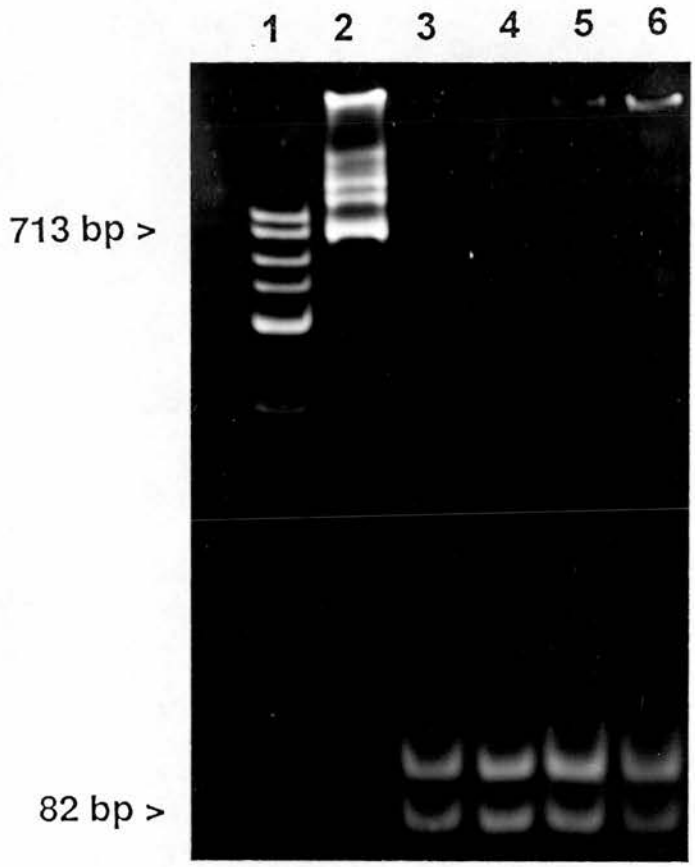


Figure 3.2 Ethidium bromide stained 5%PAGE of nested PCR amplification products of presentation marrow DNA from a patient with (DL) T-ALL with a Vδ1-D-Jδ1 rearrangement. Lane 1 contains ϕX174 DNA digested with HinfI. Lane 2 shows first round amplification product (approx. 700bp), lanes 3 to 6 show second round amplification products using the first round product as substrate with increasing magnesium concentration from 1mM (lane 3) to 2.5mM (lane 6) in 0.5mM increments. The presence of two amplification products indicates a biallelic rearrangement.

3.4.3.2 Generation and labelling of "clonospecific" probes

After the second cycle of amplification, the PCR product was subjected to digestion with the restriction enzyme FokI to separate the specific region of the probe from germline DNA sequences. The PCR product was resolved in a 4% NuSieve 3:1 agarose gel and the probe band excised with a clean razor blade. The fragment was purified by adhesion to glass beads (MERmaid kit) and radiolabelled by extension from a specific hexamer (Table 3.1) as described for labelling of bcl-2 in section 2.4.3.3.

3.4.3.3 Sequence analysis

PCR products purified as described above were sequenced as previously detailed (section 2.4.6.2) using the 3' Vδ1 amplimer as the sequencing primer.

3.4.3.4 Detection of residual disease

Patients DNA samples were amplified using the system detailed above, slot blotted onto nylon membranes as described in section 2.4.5.3. FokI digestion of PCR products was not performed. Membranes were hybridised against the patients particular "clonospecific" probe, washed stringently and autoradiographed as described in sections 2.4.5.6-8.

3.4.4 PCR Analysis of Vδ2-Dδ3 rearrangements in B-lineage ALL- generation of "clonospecific" probes.

The technique outlined for the detection of residual

disease in cALL (Yokota et al 1991a) is analogous to that described above, with the exception that primers derived from Vδ2 and Dδ3 regions are employed (Figure 3.1) as these segments are commonly recombined in B-lineage ALL.

3.4.4.1 Polymerase chain reaction analysis

PCR was performed using the primers designed by Yokota et al (1991a) listed in Table 3.1. Each reaction contained the reagents described in section 3.4.3.1. The cycling conditions are as detailed in Table 3.2.

The technique devised by Yokota also involved a nested approach, with the Vδ2 L (leader) and Jδ1 amplimers in the first set of cycles and either the Vδ2 3', Dδ3 5' or Vδ2 5', Dδ3 3' configuration in the second set of cycles for the generation of "clonospecific" probes or monitoring of residual disease respectively (Figure 3.1).

A magnesium titration to determine the optimal value was undertaken for each set of amplimers with DNA from a patient previously characterised by Southern blot analysis and 2mM $MgCl_2$ found to be the optimal value in each case. A typical titration is shown in Figure 3.3.

3.4.4.2 Generation and labelling of "clonospecific" probes

This was as detailed above (section 3.4.3.2) using the Vδ2 labelling hexamer, without the FokI digestion step.

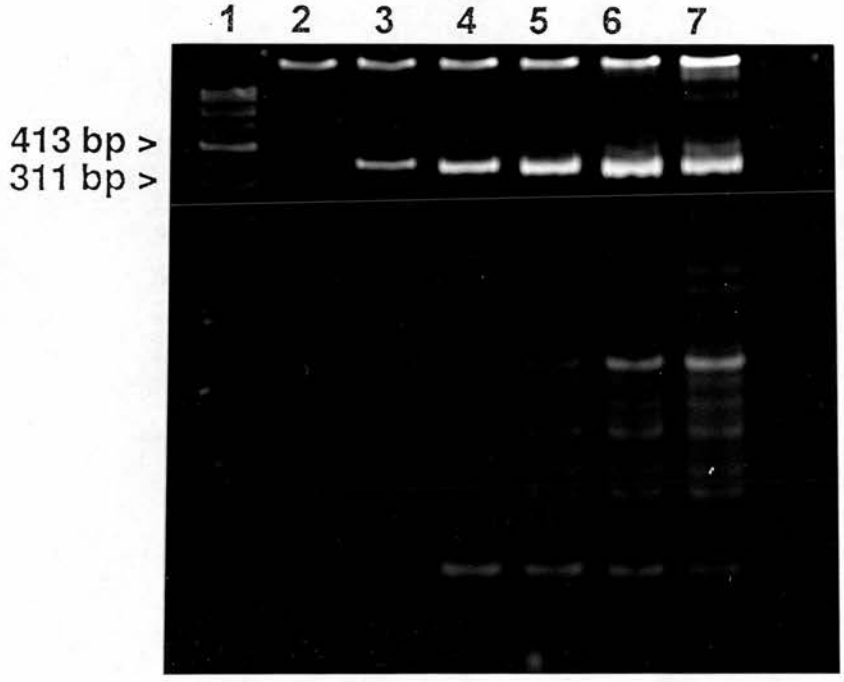


Figure 3.3 Ethidium bromide stained 5%PAGE of PCR amplification products from a cALL patient with a Vδ2-Dδ3 rearrangement. Lane 1 contains ϕX174 DNA digested with HinfI. Lanes 2 to 7 show the effect on increasing magnesium concentration from 0.0mM (lane 2) to 2.5mM (lane 7) in 0.5mM increments.

3.4.4.3 Detection of residual disease

This was as detailed above (section 3.4.3.4) with Vδ2 L and Jδ1 primers employed for the first round and Vδ2 5' and Dδ3 3' for the second.

3.4.5 PCR Analysis of Vδ2-Dδ3 rearrangements in B-lineage ALL- design of oligonucleotide probes

Due to the problem of background signals associated with probes produced directly from amplified tumour in patients known to have a Vδ2-Dδ3 rearrangement by Southern blotting ("clonospecific" probes), sequence analysis of the junctional region was performed. Further to this, a single step system was developed to minimise the time required preparing reactions, reduce problems associated with carry-over and reduce background signals. PCR conditions were as described before, using only the Vδ2 5' and Dδ3 3' primer pair in combination with the cycle conditions described in Table 3.3.

3.4.5.1 Sequence analysis

After amplifying 0.1μg tumour DNA, the product was resolved by 4% agarose gel electrophoresis and purified by adhesion to glass beads (GeneClean). Sequences were extended from the Vδ2 3' amplimer using the procedure described in section 2.4.6.2

3.4.5.2 Design of anti-junctional oligonucleotides

Due to the restricted junctional variation at incomplete Vδ2-Dδ3 rearrangements compared to that seen in Vδ1-Jδ1,

Temperature (C)	Time (s)	Number Of Cycles
94	90	
56	60	34
72	90	
94	90	
56	60	1
72	600	

Table 3.3 Cycling conditions for single step Vδ2-Dδ3 amplification.

20 nucleotide synthetic probes were designed against the junctional VN(DN)D region to maximise the specificity of hybridisation and reduce non-specific signals.

Oligonucleotide probes were end labelled, the labelling reaction consisted of 1x end labelling buffer, 10pmol probe DNA, 20μCi gamma ^{32}P dATP, 1U T4 polynucleotide kinase in dH_2O to 20μl. After incubation at 37°C for 20min, the reaction was diluted to 500μl with hybridisation buffer and the incorporation and specific activity determined from a 2μl aliquot by scintillation counting (section 2.4.3.3). An appropriate amount of probe to give $1.5x10^6$ dpm per ml prehybridisation fluid was used without further purification.

3.4.5.3 Hybridisation with oligonucleotide probes

Calculation of melting temperature (Tm) for individual probes was performed according to this formula (Sambrook et al 1989):

$$Tm=81.5+16.6(\log_{10}[Na^+])+0.41(\%G+C)-600/L$$

where $[Na^+]$ is the molarity of sodium ions (generally 0.5M), (%G+C) is the percentage G and C content and L the length of the oligonucleotide (20 bases).

Optimal hybridisation and washing conditions were determined empirically. PCR products were transferred to nylon membranes as described in section 2.4.5.3 and hybridised in glass tubes in 1.5ml of hybridisation solution at 65°C, or 5°C below the Tm if the melting

temperature of a particular oligonucleotide was below 70°C. Prehybridisation was performed for 1hr before the addition of labelled probe and further hybridisation for 1hr. Initially washing was performed for 10min at room temperature in wash 1 followed by 30min at the hybridisation temperature in wash 3.

To minimise background signals and maximise the intensity of specific binding, replicate membranes slotted with amplified tumour DNA from patient BM consisting of a 0.1% titration point and a normal DNA negative control were hybridised with 1.5 $x10^6$ cpm/ml probe (Tm 72°C). The efficiency of washing was determined by comparing standard wash conditions to washing in tetramethyl ammonium chloride (TMACl) which removes the differential binding characteristics of GC as compared to AT base pairs, hence probes of the same length will have the same Tm irrespective of base composition (Wood et al 1985). Replicate blots as described above were hybridised rinsed in wash 1 and washed for 30min in the same wash at room temperature. Membranes were washed twice in TMACl wash at 60, 61, 62 and 63°C, 60°C gave the strongest signal and the lowest background (Figure 3.4).

In order to determine if TMACl washing was more effective than washing in SDS solutions, replica blots (as above) were prepared and hybridised with the particular oligonucleotide probe. TMACl washing at 60°C was compared to washing in wash 3 at 45, 55 and 65°C which

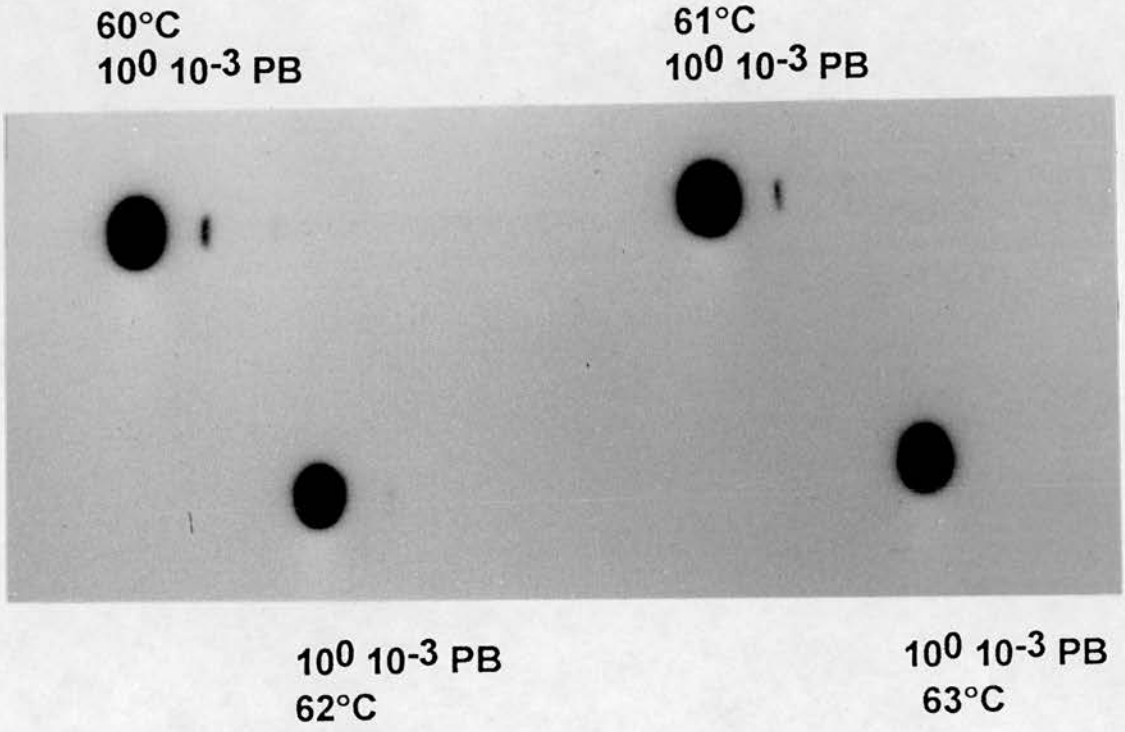


Figure 3.4 Autoradiograph showing replicate Vδ2-Dδ3 PCR amplification products from the bone marrow of a patient with cALL. PCR products were slot blotted onto nylon membranes and hybridised with a clone specific oligonucleotide probe. The replicate blots were washed using TMACl at the following temperatures 60°C, 61°C, 62°C and 63°C. Each blot consisted of 3 PCR products, undiluted tumour, 10^{-3} dilution of tumour and a pooled PB control.

demonstrated that TMACl washing resulted in the loss of specific signal compared to the 55°C wash in solution 3 (Figure 3.5).

3.4.5.4 Detection of residual disease

Patients with Vδ2-Dδ3 rearrangements were sequenced and probes designed against the VN(DN)D region. Presentation and post chemotherapy samples were amplified and 2μl PCR product slotted onto nylon membranes which were hybridised with the individual patient specific probe, washed and neoplastic cells visualised by autoradiography. In order to monitor possible binding of probes to sequences contributed by normal lymphocytes, controls consisting of pooled normal peripheral blood or bone marrow DNA were used. Applications of this technique in the monitoring of MRD are discussed in Chapter 5.

3.5 RESULTS

3.5.1 Southern blotting

As described in section 2.5.1, Southern blotting and hybridisation with DNA probes detects clonal populations of cells only when they exceed 5% of the population under study, generating bands which differ in size from the normal germline band. Hybridisation with the JδS16 probe to HindIII or BglII digested DNA results in a germline band of 6.8 and 5.2kb respectively. A clonal population bearing a Vδ1-Jδ1 rearrangement will give rise to 10kb HindIII and 5.4kb BglII bands, whereas a 7.0kb HindIII with a 10kb BglII configuration is indicative of a Vδ2-

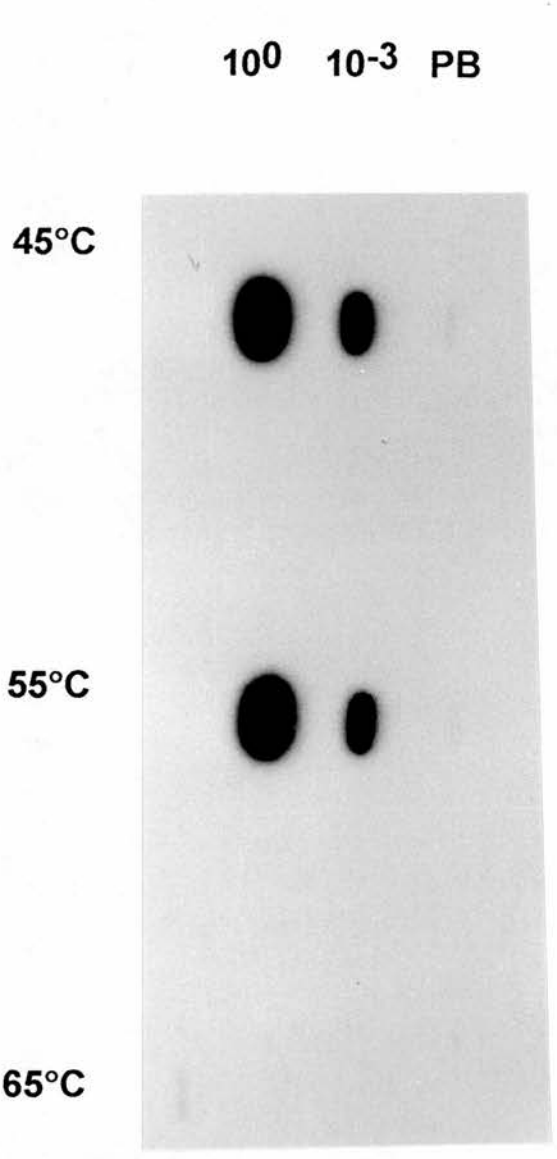


Figure 3.5 Autoradiograph showing replicate Vδ2-Dδ3 PCR amplification products from the bone marrow of a patient with cALL. PCR products were slot blotted onto nylon membranes and hybridised with a clone specific oligonucleotide probe. The replicate blots were washed using 0.1x SSC, 0.1% SDS at the following temperatures 45°C, 55° and 65°C. Each blot consisted of 3 PCR products, undiluted tumour, 10^{-3} dilution of tumour and a pooled PB control.

Dδ3 rearrangement. A BglII band of 8.5kb with no additional HindIII band is associated with a Dδ2-Dδ3 rearrangement and deletions can be detected by assessing the relative intensities of bands observed.

A total of 3 T-ALL and 22 B-lineage ALL were probed and the results summarised in Tables 3.4 and 3.5. TcR rearrangements or deletions were detected in 2 of 3 T-ALL (66%) of which one involved Vδ1-Jδ1 and in 15 B-lineage ALL (68%), of these 12 (55%) showed a Vδ2-Dδ3 configuration. Monoallelic deletions were apparent in 4 (18%) of B-lineage ALL and Dδ2-Dδ3 recombinations in 5 (23%). Of these, biallelic rearrangements (involving deletions, Vδ2-Dδ3 or Dδ2-Dδ3 rearrangements) were detected in 6 (27%). Figure 3.6 shows a group of 7 ALL (B-lineage ALL with the exception of one T-ALL in lane 5) and a normal peripheral blood DNA control digested with BglII and hybridised against the JδS16 probe. Patient 1 has a Vδ2-Dδ3 on one chromosome and a Dδ2-Dδ3 on the other, while patient 2 has a monoallelic deletion. Lanes 3 and 4 show a deletion on one chromosome, along with a Vδ2-Dδ3 rearrangement on the other. Patient 5 exhibits a biallelic Vδ1-Jδ1 configuration when digested by BglII whereas an additional band was seen in the HindIII lane, the reason for this is described below. Patient 6 is in germline configuration and patient 7 shows a monoallelic rearrangement of Vδ2-Dδ3. Lane 8 contains the control normal peripheral blood DNA.

Patient	Southern Jδ1	PCR Vδ2-Dδ3
WL	RR	+
DP	GR	+
GG	GR	+
RK	RD	+
BM	GR	+
PM	GG	-
TM	GR	+
CC	GG	-
GH	GG	-
FP	GR	-
AM	GG	-
CAW	RR	+
EC	GR	+
VH	GG	-
LH	RD	-
KM	GG	-
MS	GG	-
HS	RD	+
DM	GG	-
LW	GG	-
MC	GR	+
CA	GD	+
DT	GR	+
YD	ND	-
GC	ND	-
AM	DR	-
FW	GG	-
DR	GR	ND
YD	ND	-
GC	ND	-
HM	ND	+
SG	ND	-
SW	ND	-
SM	ND	+
LR	ND	+
MK	ND	+
CA	ND	+/+
KM	ND	+
JAM	ND	+/+

Table 3.4 Comparison of Jδ1 Southern blotting and Vδ2-Dδ3 PCR results in B-cell ALL patients. Germline configuration is represented by G, rearranged by R and deletion by D. The presence of a PCR marker is indicated by a + symbol.

Patient	Southern Jδ1	PCR Vδ1-Jδ1
DL	RR	+/+
EP	DD	-
CB	GG	-
AS	ND	-
KK	ND	-
LO	ND	-
EE	ND	-
GH	ND	-

Table 3.5 Comparison of Jδ1 Southern blotting and Vδ1-Jδ1 PCR results in T-cell ALL patients. Germline configuration is represented by G, rearranged by R and deletion by D. The presence of a PCR marker is indicated by a + symbol.

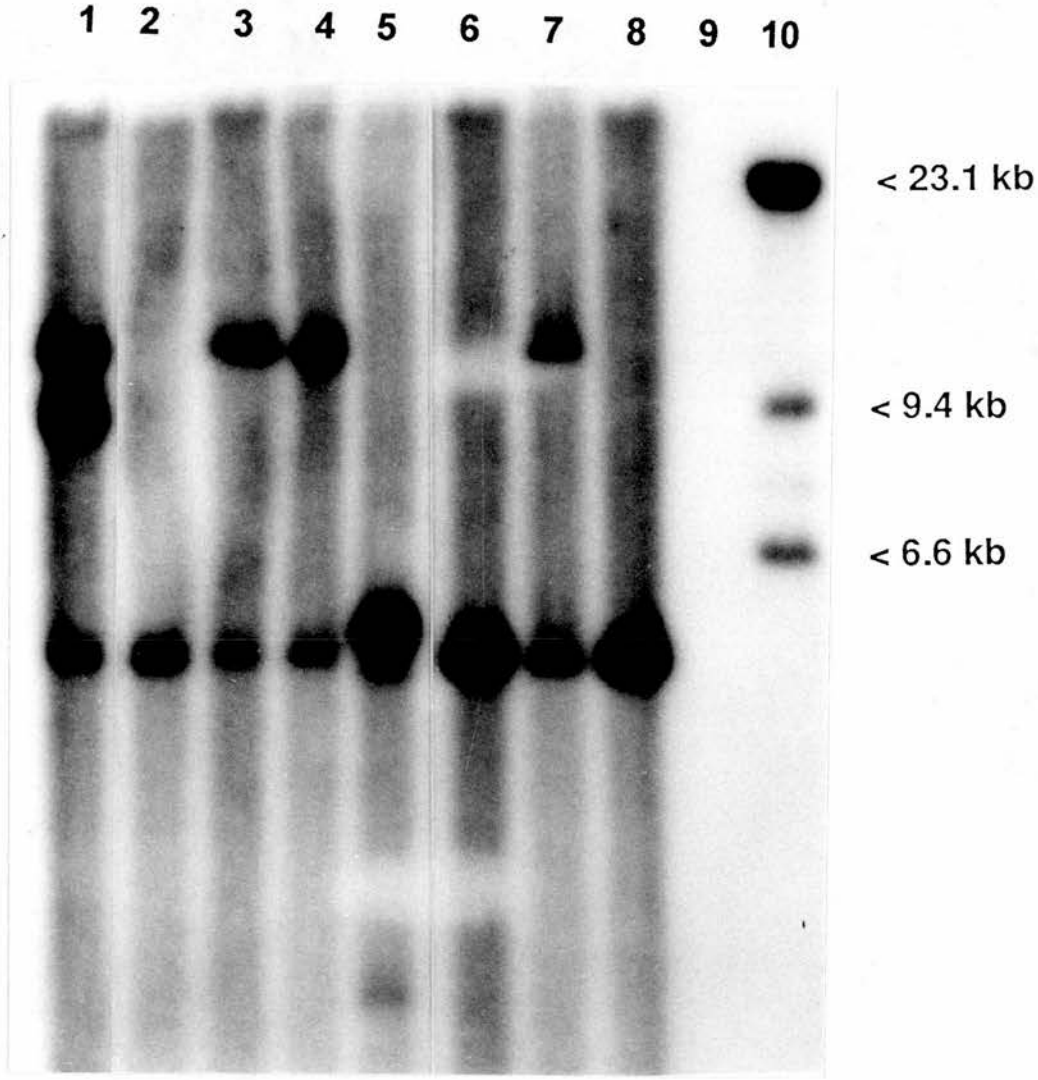


Figure 3.6 Autoradiograph of a Southern blot of presentation marrows from 6 patients (lanes 1-4,6,7) with cALL and one patient with T-ALL (lane 5). Control DNA (pooled normal PBL) was run in lane 8. The DNA was digested with BglII and hybridised with probe JδS16. Lane 10 contains a bacteriophage lambda digested with HindIII size marker. Germline configuration is indicated with a 5.0kb band, Vδ2-Dδ3 rearrangement by a 10.0kb band (lanes 1,3,4,7), Dδ2-Dδ3 (lane 1) by a 9.0kb band and Vδ1-Jδ1 by a 5.3kb band (lane 5).

Ten patients with T-lineage NHL were screened with the JδS16 probe and none demonstrated the presence of rearrangements involving the Jδ1 segment.

Of 16 AML patients analysed, 2 (12.5%) showed Jδ1 segment usage, although none showed bands indicative of either Vδ2-Dδ3 or Vδ1-Jδ1 rearrangements hence were not amenable to PCR analysis. This suggests that Vδ3, Vδ4, Vδ5 or Vδ6 had rearranged to Dδ3 or Jδ1.

3.5.2 PCR analysis of Vδ1-Jδ1 rearrangements

3.5.2.1 Generation of "clonospecific" probes

A total of 8 T-ALL patients were screened by PCR for the presence of such a rearrangement, though only one (patient DL) demonstrated amplification products in the expected 100bp range. Bands were seen (Figure 3.7, lane 2) at 100 and 120bp indicating a biallelic rearrangement, confirming the Southern blot result (patient 6 above). Also shown in Figure 3.7 is the FokI digest of these products (lane 1), in which residual non- digested material is apparent in addition to the newly generated "clonospecific" probes. The piece of DNA digested from each band will be of the same size and comigrate, forming the lightest fragment observed. Serial dilutions of the presentation DNA in pooled peripheral blood DNA were amplified and probed, indicating sensitivity at the 10^5 level (Figure 3.8)

3.5.2.2 Sequence analysis

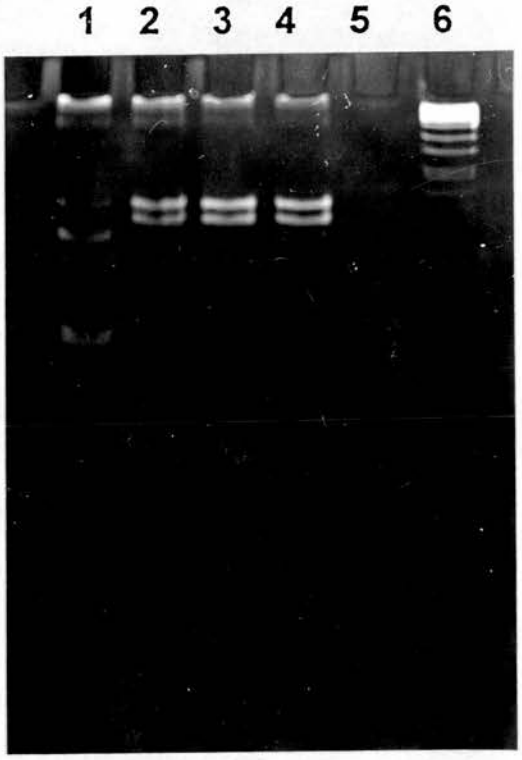


Figure 3.7 Ethidium bromide stained 5% PAGE gel of Vδ1-Jδ1 PCR amplification product from a patient with T-ALL. Lane 6 contains ϕX174 digested with HinfI. Lanes 2-4 contain the second cycle product using 3' Vδ1 and Jδ1 5' primers. A double band indicates a biallelic rearrangement. Lane 1 contains the second cycle PCR product digested with FokI generating clonospecific probes. The 2 upper bands contain undigested PCR products, the 2 middle bands contain the clonospecific probes and the lightest band contains the fragment excised by FokI digestion.

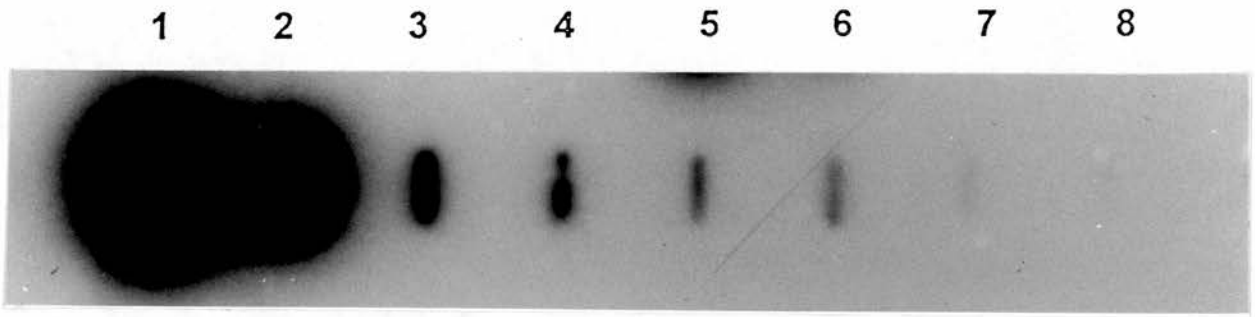


Figure 3.8 Autoradiograph of Vδ1-Jδ1 amplification products. Tenfold serial dilutions of DL DNA in normal pooled PBL DNA (lanes 1-7) were hybridised against the "clonospecific" probe. Lane 8 contains control PBL DNA.

The 2 bands described above were sequenced, extending from the Vδ1 3' primer to determine the degree of variability at this locus (Table 3.6). The junctional regions were found to span 44 and 30 nucleotides, with selection of multiple D segments, deletion of germline information and extensive N nucleotide insertion apparent. Probes derived from this region would be highly clone specific. Interestingly, the additional HindIII band observed on Southern blot analysis is explained by the creation of a HindIII recognition site in the VND region of one of the alleles resulting in a lighter band. An in frame TAG stop codon was formed in one of the junctional regions.

3.5.3 PCR analysis of Vδ2-Dδ3 rearrangements- generation of "clonospecific" probes

A subgroup of 4 Vδ2-Dδ3 positive ALL patients identified by Southern blotting were studied further by PCR. After 2 sets of amplification cycles, bands in the expected 100bp size range were obtained although discrete bands were also apparent in a pooled normal peripheral blood control. However successful amplification was not obtained in all patients. Figure 3.9 shows 2 patients and a normal DNA control after nested (lanes 2-4) amplification. Patient 1 (lane 2) demonstrates a PCR product, however only an extremely faint band can be seen in patient 2 (lane 3). A band is also visible in the normal control DNA (lane 4). It is apparent using the technique described by Yokota (1991a) that some patients

	Vδ1	N	Dδ1	N	Dδ2	N	Dδ3	N	Jδ1
DL 1	GGAACT	AAGCTTA	GAA	GTG	TCCT	GCC*TAG*	CTGGGGGAT	TCCCAT	ACAC
DL2	GGAACT	AGATCATCTGG					CTGGGGGATA	T	ACAC

Table 3.6 Nucleotide sequence of TcR Vδ1-Jδ1 rearrangements from a patient with T-ALL. A HindIII recognition site is underlined and palindromic nucleotides are indicated in bold type. An in frame TAG stop codon is shown in italic type.

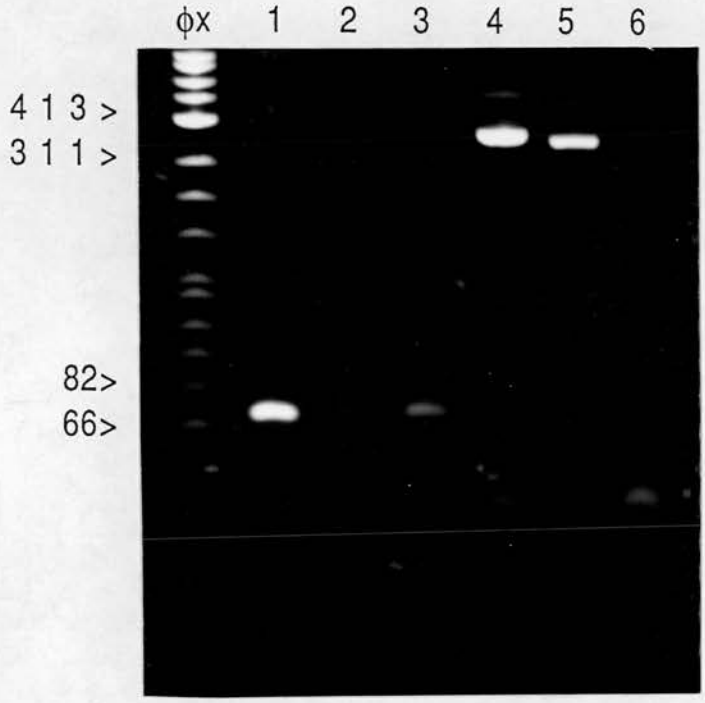


Figure 3.9 Ethidium bromide stained 5%PAGE of TcRδ PCR amplification products from 2 cALL patients. The first lane contains ϕX174 DNA digested with HinfI. Lanes 1 and 2 show the second round amplification products using the Vδ2 3' and Dδ3 5' amplimers (the first round was performed with the Vδ2 L and Jδ1 amplimers) with a pooled peripheral blood (PBL) control in lane 4. Lanes 4 and 5 shows amplified DNA from the same patients using a single step PCR amplification with amplimers Vδ2 5' and Dδ3 3'. A PBL control amplified with the same primers is shown in lane 6.

who should be amenable to PCR analysis from Southern blot analysis data cannot be used for the generation of "clonospecific" probes. Further to this, it was evident that a total of 70 cycles of amplification increased the intensity of background signals which may lead to false positive results. This is confirmed by Figure 3.10 which shows 3 patients and a negative control amplified by the nested procedure and probed with the "clonospecific" probe derived from the patient in lane 1. A strong hybridisation signal is seen in all lanes, even after a 65°C 0.1xSSC stringent wash, indicating a high degree of non-specificity in the hybridisation of the probe to tumour DNA.

3.5.4 PCR analysis of Vδ2-Dδ3 rearrangements- design of oligonucleotide probes

3.5.4.1 Application of a single step amplification system

Of 36 B-lineage ALL patients amplified using a single step system, 19 (53%) were PCR positive, including all of those patients previously characterised as Vδ2-Dδ3 by Southern blotting (Table 3.4). Two patients (6%) demonstrated 2 distinct bands, in a biallelic rearrangement. Figure 3.11 shows a sample of 5 patients with the bands of varying size in the expected 340-380bp range, along with a negative control DNA in which discrete bands are not apparent. The patient described above who did not amplify using the nested approach was amplified using the single step system (Figure 3.9). Whereas with the nested system no band could be seen

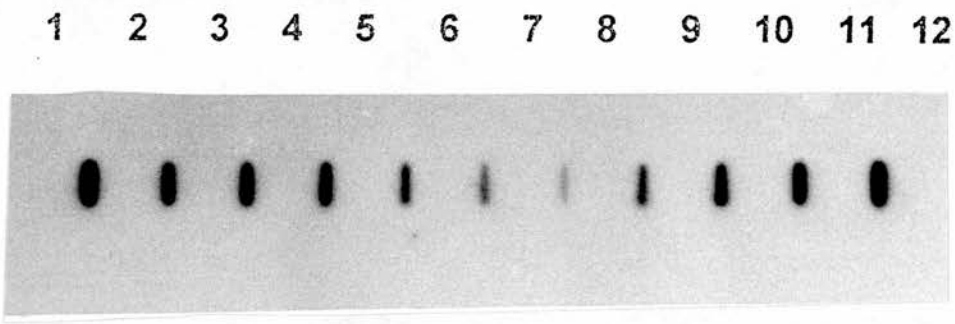


Figure 3.10 Autoradiograph of Vδ2-Dδ3 nested amplification products of 3 patients and normal PBL hybridised with a clonospecific probe derived from patient GG. Slots 1 and 2 contain products from patients DP and WL, slots 3 and 4 contains PBL and marrow from patient GG. Slots 5 to 10 contain products from DNA samples obtained from patient GG during treatment. Lane 11 contains amplified pooled normal PBL with lane 12 containing a control with no template DNA. The membrane was subjected to stringent washing prior to autoradiography.

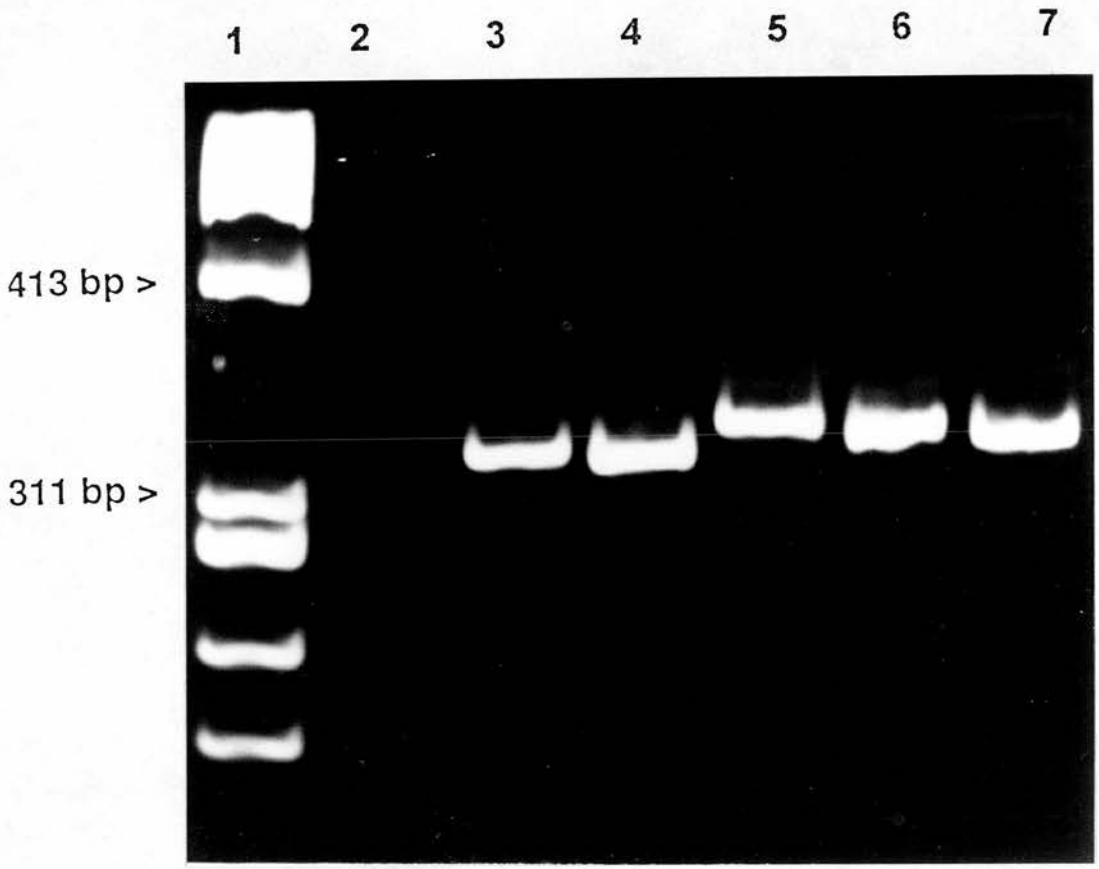


Figure 3.11 Ethidium bromide stained 5% PAGE gel of Vδ2-Dδ3 PCR amplification products from 5 patients with cALL. Lane 1 contains a ϕX174 digested with HinfI size marker. Lane 2 contains a pooled PB control. The size of the PCR product varied from 340 to 380 bp.

(Lane 3), with the single pair of more distal primers a distinct band is present (Lane 6) suggesting that one of the inner primer sites was deleted. Lanes 2 and 5 show a patient who amplifies with both systems, with negative control DNA in lanes 4 and 7, nested and single step respectively.

3.5.4.2 Sequence analysis

The Vδ2-Dδ3 junctional sequence from 18 patients (11 children and 7 adults) was determined (Table 3.7) with a typical autoradiograph shown in Figure 3.12. One of these patients exhibited a biallelic pattern but only one allele was sequenced. The junctional region varied in size from 0-13 nucleotides, with an average length of 7. Varying degrees of deletion from both the 3' terminus of Vδ2 and 5' of Dδ3 germline sequences was apparent ranging from 0 to 15 and 0 to 24 nucleotides respectively with only one patient showing no deletion at either terminus. N nucleotide insertion, ranging from 0 to 13 nucleotides (average 5.5) was seen in 15 of 18 patients and were were 79% GC rich. Dδ segment involvement was ascribed on the basis of 3 consecutive nucleotides from either Dδ1 or Dδ2 germline segments and was apparent in 6 junctions. P nucleotides were also observed at 5 intact germline boundaries. Three patients had large deletions which removed part of the sequence complementary to the Dδ3 amplimer used by Yokota et al for the generation of "clonospecific probes". This explains why "clonospecific" probes could not be generated from a patient

Vδ2	CTGTGCCTGTGACACC		
Dδ1		GAAATAGT	
Dδ2		CCTTCCTAC	
Dδ3			ACTGGGGGATACG
intron			**CACAGTG**CTACAAA
WL	CTGT**GCCTGTG**	**CCACAG**	**GGGGGAT**ACG CACAGTGCTACAAA
DP	CTGTGCC**TGTGACAC**	**GCA**	**GGATACG CA**CAGTGCTACAAA
GG	CTGTGCCTG**TGAC**	**CCGACCCCCATGG**	**TG**GGGGATACG CACAGTGCTACAAA
BM	**C**	**CGTCGCCGG**	**GTGCTAC**AAA
DT^p	CTGTGCCTGT**GACAC**	**TCCCCATTGT***	**ACTGG**GGGATACG CACAGTGCTACAAA
DT^r	CT**GTGCCTG**	**AGAAGG**	**GGGGGAT**ACG CACAGTGCTACAAA
DT^r	CTGTGCCTG	AGAAGG	GGGGGATACG CACAGTGCTACAAA
TM	CTGTGCCT	CCTTCGGAC	CG CACAGTGCTACAAA
HS	CTGTGCCTGTGACACC	CCTT*	ACTGGGGGATACG CACAGTGCTACAAA
RK	CTGTGCCTG**TGAC**	**GTGTCTCCGTC**	**GGGAT**ACG CACAGTGCTACAAA
EC	CTGTG**CCTGTGACAC**		**AAA**
CA	CTGTGC**CTGTGAC**	**CGA**	**GGGATACG CA**CAGTGCTACAAA
CW	CTG**TGCCTGTGACACC**	***GGGGGG**	**TG**GGGGATACG CACAGTGCTACAAA
HM	CTGTGCCTGTGACAC	GGGG	CTGGGGGATACG CACAGTGCTACAAA
MC	CTGTGCCT**GTGACAC**	***GGCCTT**	**TACG CAC**AGTGCTACAAA
JM	CTGTGCCTGTGA	GACCCAC	GGGGATACG CACAGTGCTACAAA
SM	CTGTGCCTGTGACAC	TCCCCTGGGGG	TGGGGGATACG CACAGTGCTACAAA
LR	CTGTGCCTGTG	CCCCCCGGA	ACAGTGCTACAAA
MK	CTGTGCCTGTG	GTCTGT*	ACTGGGGGATACG CACAGTGCTACAAA
KM	CTGTGCC	CCCC	GGGGGATACG CACAGTGCTACAAA

Table 3.7 TcR Vδ2 to Dδ3 gene rearrangements in ALL. Germline Vδ2, Dδ1, Dδ2 and Dδ3 sequences are indicated at the top. Homology with Dδ3 sequences are underlined and palindromic nucleotides are marked with an asterisk. The Dδ3 recombination heptamer is shown in bold. A 20 base probe was designed for patient EC, although the 3' component of the sequence is not shown on the table, likewise the most 5' nucleotides from patient BM are not shown. In patient CW, the probe was designed from a more 5' region to reduce a very high G bias. The sequence shown in bold within the Dδ3 germline sequence represents the RSS. Elsewhere bold type indicates probe location.

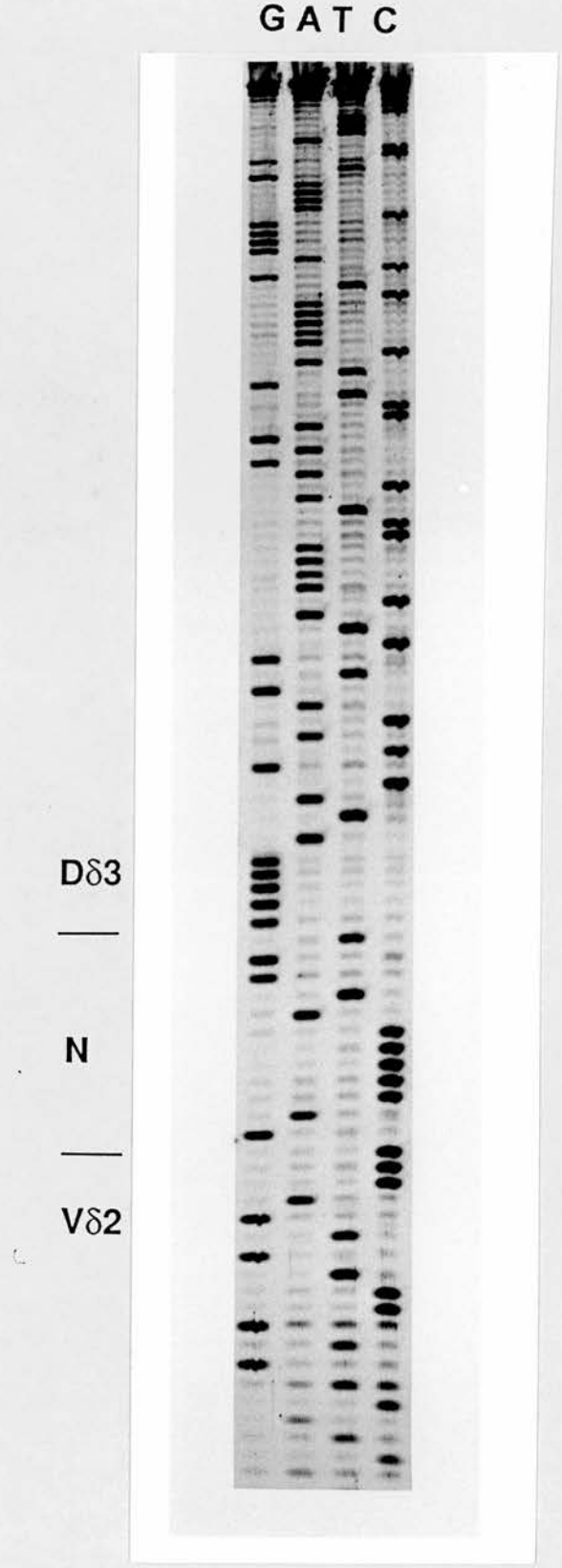


Figure 3.12 Autoradiograph of a 6% denaturing PAGE gel for sequencing PCR products from a cALL patient with a Vδ2-Dδ3 rearrangement. Products were sequenced using an internal sequencing primer. Termination reactions were loaded in the order G, A, T and C.

characterised as having a Vδ2-Dδ3 rearrangement by Southern blotting. One patient (BM) had the Vδ2 labelling hexamer site deleted and another (KM) lost one nucleotide from this site. These findings suggest that production of "clonospecific" probes would either be difficult or impossible in more than 10% of patients. The structure of the junctions has implications for oligonucleotide probe design as discussed below.

3.5.4.3 Probe design

To exploit the diversity contributed by D segment involvement, N nucleotide insertion and germline deletion, oligonucleotide probes of 20 nucleotides in length were designed against the unique junctional regions, flanked by germline Vδ2 and Dδ3 sequences. Probes specific for 10 patients were synthesised in total, indicated by bold type in Table 3.7. Replicate slot blots consisting of the amplification products from an initial group of 5 patients and one pooled normal control DNA were probed in turn with oligonucleotide probes derived from each patient (Figure 3.13). Specific hybridisation of each probe was seen, with no signal apparent in the negative control or cross hybridisation between patients.

3.5.4.4 Probe sensitivity

Serial tenfold dilutions of presentation DNA from one patient were made in control DNA were amplified in triplicate and resolved by PAGE (Figure 3.14). PCR

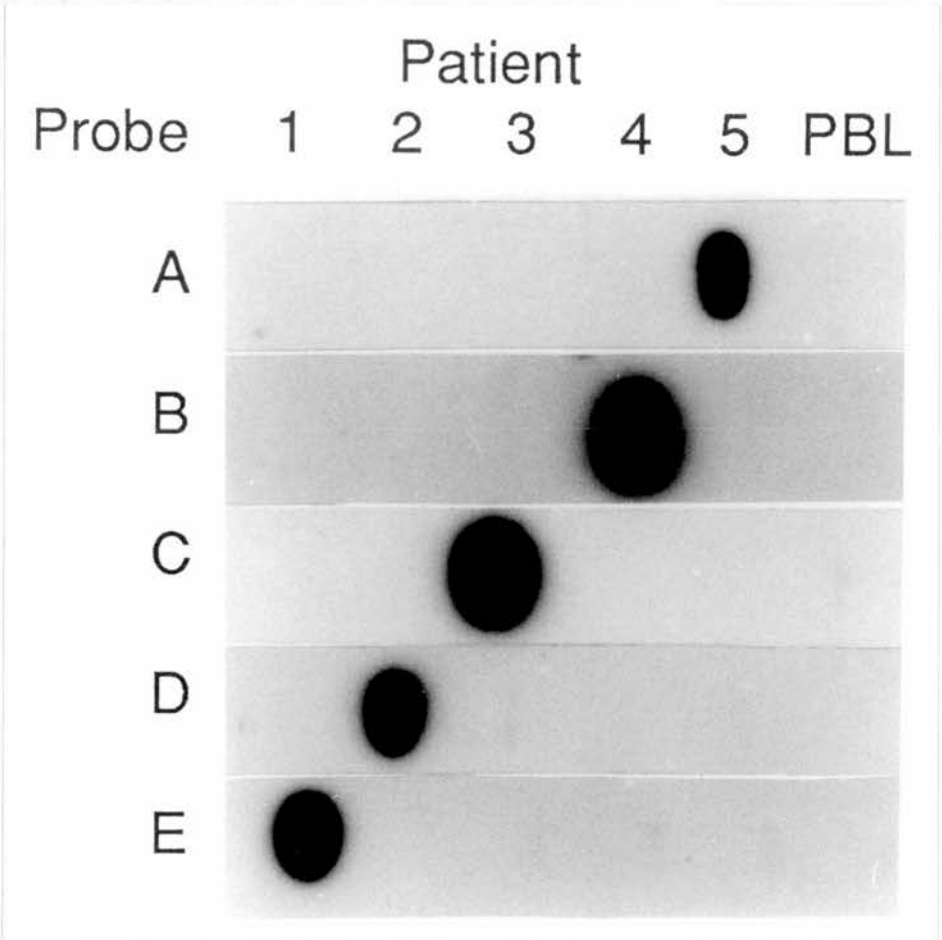


Figure 3.13 Autoradiograph of Vδ2-Dδ3 PCR amplification products of tumour DNA from 5 patients and normal pooled PBL. Replicate slot blots of PCR amplification products were hybridsed with clone specific probes (probes A-E) derived from each patient at presentation.

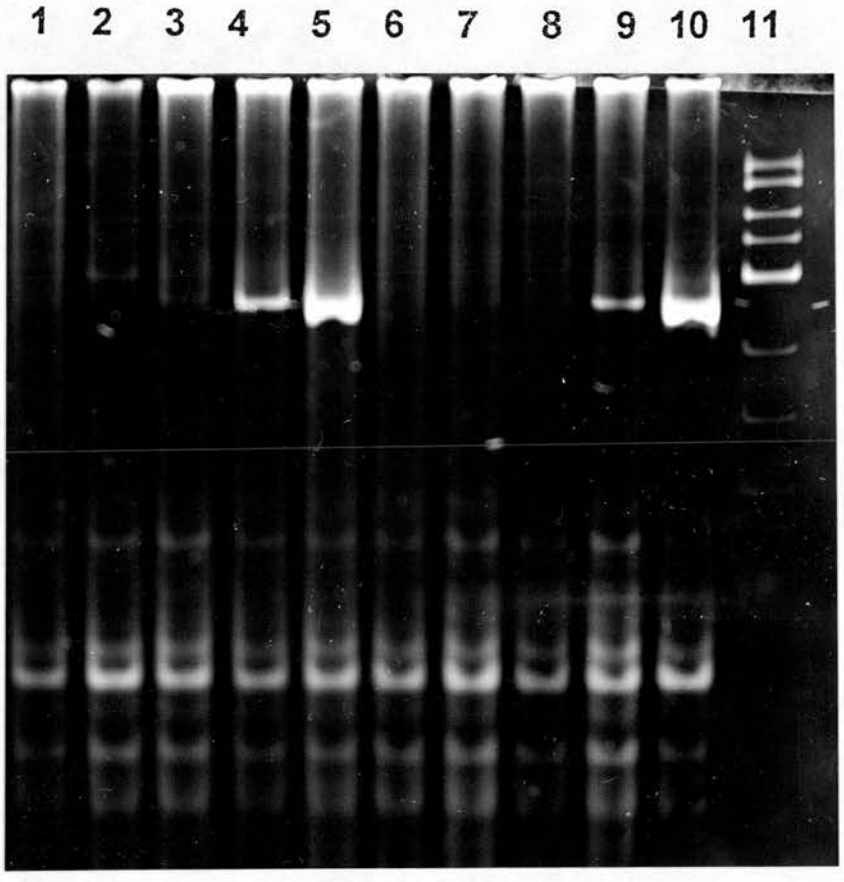


Figure 3.14 Ethidium bromide stained 5% PAGE gel of Vδ2-Dδ3 PCR amplification products from a patient with cALL. Lane 11 contains a ϕX174 digested with HinfI size marker. Tenfold serial dilutions (10^{-4} to 10^{0}) of presentation bone marrow DNA were amplified in duplicate (lanes 1-5, 6-10). A single band of 350bp indicates the presence of a clonal Vδ2-Dδ3 rearrangement.

products were slot blotted onto nylon membranes and probed with the relevant probe (Figure 3.15). A decreased signal intensity was seen with subsequent dilutions, lane 5 (10^4 level) being the limit of sensitivity. However due to the variation in junctional sequences, sensitivity will be dependent on probe constitution. Further titrations are discussed in Chapter V.

3.6 DISCUSSION

In order to determine the incidence of TcRδ-chain rearrangements in haematological disorders and the potential application of this locus as a specific marker for the detection of residual leukaemia, both Southern blot and PCR based systems were investigated.

Southern blot analysis with a TcRδ-chain joining region probe was applied to a range of disorders to study the association of TcRδ rearrangements with haematological malignancies in samples collected at presentation. Hybridisation with the JδS16 probe identified one of 3 T-ALL patients (33%) with a Vδ1-Jδ1 rearrangement, compared to a published incidence of 25% (Hansen-Hagge et al 1989). Approximately 10% of patients with AML have been reported to show δ-chain rearrangements (Adriaansen et al 1991) and were found in 2 of 16 patients in this study (12.5%). However, of these none demonstrated a configuration amenable to further analysis, confirmed by the report of Fontenay et al (1990) who detected no Vδ1-

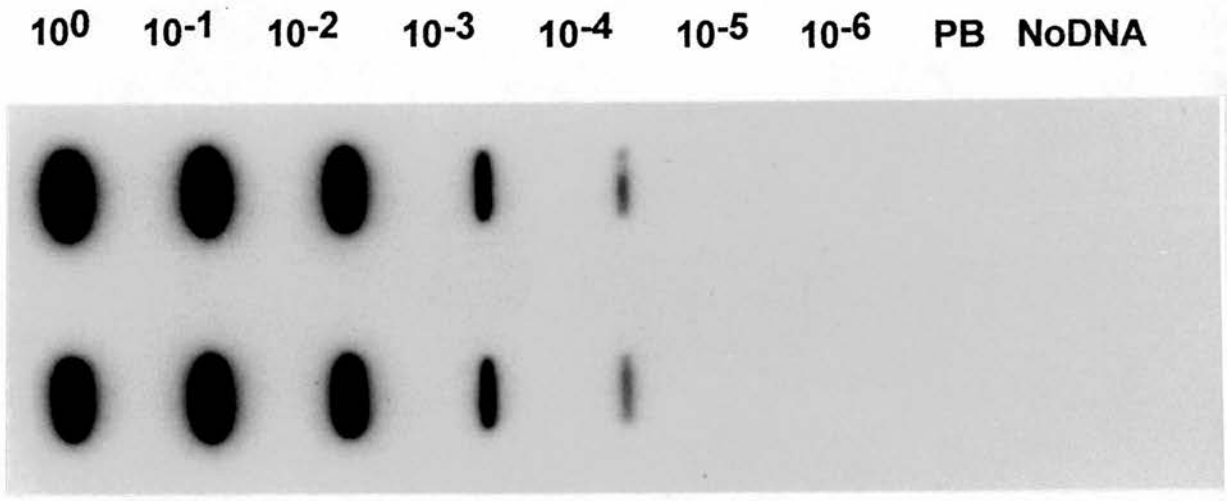


Figure 3.15 Autoradiograph of Vδ2-Dδ3 PCR amplification products of tumour DNA from a patient with cALL diluted in pooled PBL DNA and hybridised to the patient's clone specific probe. Dilutions range from 10^{-1} to 10^{-6} with undiluted tumour DNA in lane 1. Lane 8 contains pooled PBL DNA.

Jδ1 or Vδ2-Dδ3 rearrangements. One of 10 T-NHL also displayed a TcRδ rearrangement, in concordance with the published incidence, though again the gene segments involved could not be analysed further by the Vδ1 or Vδ2 based PCR systems. The majority of T-NHL δ-chain rearrangements are deletions (Kanavaros et al 1991) but the quality of the lymph node biopsy specimens available made accurate assessment of band intensity difficult, so such events have not been recorded here. The group of most interest however was that of B-lineage ALL, of the 22 probed 15 (68%) showed mono- or biallelic TcRδ-chain rearrangements, 80% of which were Vδ2-Dδ3 events. TcR Dδ2-Dδ3 recombinations and monoallelic deletions were also detected. The previously reported overall incidence of rearrangements at this locus range from 70 to 81%, of these the majority were reported to be Vδ2-Dδ3 events (Hara et al 1988, Biondi et al 1990). A total of 255 patients were investigated in this and other studies, with a 50% overall incidence of Vδ2-Dδ3 rearrangements (Hara et al 1988, Yokota et al 1991a, Yano et al 1991, Biondi et al 1990).

As the junctional diversity shown at the TcRδ locus is extremely high, it provides an excellent target for PCR analysis (Hansen-Hagge et al 1989). Generally screening by PCR involves the use of a single TcRδ primer pair, determined by the disorder under study, Vδ1-Jδ1 in T-ALL and Vδ2-Dδ3 in B-lineage ALL. The diversity encoded at the Vδ1-Jδ1 junction enables the production of sensitive

"clonospecific" probes in 25% of patients with T-ALL. However, only one of 8 patients (12.5%) in this study was amenable to analysis by this technique. In this case, probes were derived from two rearrangements and sensitivity at the 10^{-5} level was achieved. As one microgram of DNA was used as the substrate for the reaction, this represents the detection of one copy. Sequence analysis demonstrated the enormous diversity providing this level of specificity with 2 junctions of 44 and 30bp characterised. Deletion of germline segments, N insertion and multiple D segment selection result in extremely diverse sequences, with P nucleotide involvement seen at intact germline boundaries. One of the sequences contained an in frame stop codon indicating that it would not encode a functional allele whereas the other allele may code for a functional gene but as the leukaemic clone did not express a $\gamma\delta$ TcR neither allele would encode a cell surface molecule. Neale et al (1991) screened a group of T-ALL with a series of Vδ1, Vδ2 and Vδ3 5' primers and Jδ1, Jδ2 and Jδ3 3' primers, enabling amplification in 76% of cases, hence this approach can be extended to a larger group of patients and may also be of value in the study of AML.

Of the 36 patients with B-lineage ALL amplified, Vδ2-Dδ3 associated bands were found in 19 (53%) and these patients should therefore be amenable to analysis with "clonospecific" probes (Yokota et al 1991a). However, when this approach was undertaken, problems were

encountered. Unlike the Vδ1-Jδ1 rearrangement, Vδ2-Dδ3 junctions arise from incomplete recombinations, hence are less diverse (Breit et al 1991). "Clonospecific" probes derived from these regions vary in sensitivity, necessitating the study of dilution series on an individual patient basis. The results obtained with these probes were unsatisfactory, due to the relatively nonspecific nature of the "clonospecific" probe resulting from the inclusion of germline sequences. Probes derived from tumour DNA amplified at presentation may also contain sequences from normal polyclonal lymphocytes which would also reduce specificity. In addition, one patient shown by Southern blot analysis to have a Vδ2-Dδ3 rearrangement did not amplify with the inner primer pair.

In order to minimise these problems, several steps were taken. Initially a single step amplification was introduced, reducing the number of cycles from 70 to 35 in order to minimise artefacts introduced by excessive cycling, reduce the signal contributed by polyclonal lymphocytes and remove the possibility of cross-contamination associated with nested PCR. Nested PCR involves using the products of one reaction as substrate for another, hence the preparation of the second set of reactions requires a separate area and extreme care with handling vessels and reagents as the introduction of even a single copy of contaminating DNA may lead to a false positive result. A single step approach is therefore less prone to contamination, requires fewer handling areas and

reduces preparation and running time. Further to this it was decided to sequence the PCR products and design shorter probes derived from the junctional region which would contain less germline sequence, hence would be less likely to match polyclonal sequences.

Sequence analysis showed a variety of junctional regions ranging in length from 0 to 13bp. Diversity was contributed by extensive germline deletion, N insertion and D segment selection. The majority of the junctional sequence was contributed by N nucleotides which constituted 75% of this region, with 18% from D selection. Probes derived from less diverse regions may be more likely to match a polyclonal lymphocyte but generally considerable diversity was apparent and oligonucleotide probes were designed which flanked the junctional sequence. The GC bias of terminal transferase would result in extremely GC rich probes in some instances which may reduce probe sensitivity. Interestingly, sequence data from other groups (Yokota et al 1991a, Greisinger 1992) showed a high frequency of intact 5' Dδ3 termini leading to the suggestion that such a configuration could be a feature of ALL. However this was not confirmed by this study, in which only 3 of 18 Dδ3 segments were intact. This discrepancy may be due to the selection of amplimers close to the Dδ3 gene, resulting in primer site deletion in a proportion of cases, therefore underestimating the frequency of patients with extensive deletions. Such deletion removed

the 5' Dδ3 primer site in 3 patients, explaining why one patient (BM) was shown to have a Vδ2-Dδ3 rearrangement on Southern analysis but would not amplify with the nested primer set to generate a "clonospecific" probe. Relocating the Dδ3 primer to a more 3' site could extend the applicability of "clonospecific" probes however this would increase the involvement of germline DNA in a supposedly patient specific probe which could further reduce sensitivity. Likewise the "clonospecific" probe labelling hexamer had been partially or completely deleted in 2 patients but a 5' relocation of this hexamer would also reduce specificity.

Probes 20 nucleotides in length were designed to provide specificity and facilitate hybridisation conditions in the 65°C range, exploiting the diversity provided by trimming, D usage and N insertion. The N(D)N region was selected as the centre of the probe, with the remaining nucleotides derived from equal amounts of 5' Vδ2 and 3' Dδ3 sequences. The use of oligonucleotide probes rather than "clonospecific" probes of varying length facilitates allele specific washing. Conditions were selected to destabilise non-specific matches between the probe and target, reducing background signals. Initially washing in a tetra alkyl ammonium salt which weakens G-C bonds therefore minimising the effect of base constitution on hybridisation kinetics was investigated (Wood et al 1985). This would enable probes with different nucleotide sequence to be washed with uniform conditions. However

empirical studies showed that optimum wash temperature remained probe specific, hence a standard SSC wash was pursued, with a simple 55°C wash step adequate to remove signal from negative control DNA lanes. This simplified discrimination of rare leukaemic signals from background populations.

The selection of representative control DNA was crucial, polyclonal sequences representative of "normal" background rearrangements was required to show that probe binding was specific to sequences associated with neoplastic cells. This was approximated by pooling peripheral blood DNA from a series of individuals and running these with each set of amplification reactions. Controls containing no template DNA were run to monitor possible contamination.

Sensitivity at the 10^{-5} level was achieved when serial tenfold dilutions of tumour DNA (assumed to be 100% malignant blasts) were made in polyclonal DNA derived from pooled normal mononuclear cells. As one microgram of DNA is derived from approximately 10^5 cells this indicates the detection of one TcRδ chain rearrangement, the physical limit of sensitivity. However the intensity of background signals contributed by normal lymphocytes will depend on the degree of junctional variability therefore sensitivity will vary on a sequence dependent basis, explaining the 10^{-4} limit observed for some probes.

When compared to the relative ease of "clonospecific" probe production, sequencing may appear to be both laborious and expensive, however the gain in sensitivity, ease of interpretation and number of patients amenable to analysis renders it advantageous. All the patients found by Southern analysis to have Vδ2-Dδ3 rearrangements produced discrete PCR products which could be sequenced, likewise the negatives yielded no discrete amplification. Southern analysis is of value in the detailed analysis of rearrangements as a single probe can investigate a variety of recombination events which would require a series of PCR amplimers. However, when a single event is studied, PCR analysis can be performed rapidly and is relatively inexpensive.

Biallelic rearrangements at this locus have been detected frequently by Southern blotting (Yokota et al 1991a), in this study 2 patients analysed by PCR showed 2 discrete Vδ2-Dδ3 PCR products. If biallelic bands are closely spaced, sequencing can be difficult. If they cannot be resolved by electrophoresis the bands can be pooled and sequenced from both directions and the sequences subtracted though this is not possible if more than 2 sequences are obtained, in which cases cloning would be required. Biallelism has implications for probe design as one allele may be lost at relapse, hence for prospective analysis, probes should be designed against each

junctional sequence to reduce the risk of not detecting persisting disease.

The frequent involvement of Dδ3 sequences in the junctional regions, combined with the occasional detection of Dδ2-Dδ3 rearrangements on Southern blotting suggests a hierarchy of rearrangements, with Dδ2 to Dδ3 recombination the initial event, followed by Dδ2-Dδ3 to Vδ2 joining. This partial complex could then join to a Jδ segment to form a complete δ-chain rearrangement (Hara et al 1991) or alternatively recombine to downstream Jα elements before formation of an α-chain rearrangement (Yokota et al 1991a). However any subsequent rearrangement would be blocked by the frequently observed deletion of the recombination heptamer located immediately 3' to Dδ3.

The amplification of immune receptor gene rearrangements as markers of disease provides a simple method for screening large numbers of patients. The extension of TcR analysis to include the γ-chain genes would increase the number of patients amenable to analysis and provide corroboration of results. However there are problems associated with TcR γ chain analysis as amplification of the gamma-chain genes involves the use of 6 family specific 5' V and 3 3' J amplimers (d'Auriol et al 1989) as opposed to the single primer pairs required for TcRδ chain analysis. This requires a pre-screening step by Southern blotting to identify which families are involved

prior to amplification. Taylor et al (1991a) described a simplified method whereby restriction enzyme digestion was employed to determine V γ usage after amplification using a single pair of consensus amplimers. Consensus primers were also used by Tycko et al (1989) to generate a product which was subsequently characterised for V family usage. Residual disease was then assessed by amplification from a 5' Vgamma family specific and 3' patient specific junctional primer, though sensitivity was not reported. Velkeen et al (1991) described an "RNase" protection assay whereby amplified TcR γ junctions were transcribed from a T7 promoter linked to one amplimer and the resulting RNA used to probe remission amplification products. Mismatches were removed by the action of RNase and sensitivity at the 10^{-5} level was reported. This method avoids sequencing, but may suffer from the problems associated with "clonospecific" probes discussed above.

The location of the TcRδ locus within the TcRα-chain implies that recombination of TcRα subunits will delete any previous rearrangement, hence δ-chain rearrangements occurring prior to that of the α-chains cannot be studied. This may make TcR γ a more prevalent marker but MacIntyre et al (1989) suggested that the presence of polyclonal T-lymphocytes in samples under study could diminish the sensitivity of TcR γ derived probes.

It is essential to the study or MRD that tumour specific

markers do not change with disease progression, a phenomenon termed clonal evolution as this could lead to false negative assessment of disease persistence. It has been suggested that both the TcR γ and δ loci provide stable markers for study as alterations from presentation to relapse in only 10 to 20% of patients (van Dongen 1992), which compares favourably to the rate of evolution reported at the IgH locus (Beishuizen et al 1991a), although this will be discussed in greater detail in chapter IV.

Other techniques, such as the amplification of chromosomal translocations have been used, and these events may provide extremely stable markers of disease as they occur at an early stage of tumourigenesis. However as the incidence of any specific translocation is low, if all the most frequently occurring lesions are pooled, only 20% of patients could be studied, therefore pools of many primers would be required to screen relatively few patients. Further to this, many translocations have not been characterised at the molecular level and in those which have, the size of the translocation breakpoints usually requires an RNA substrate for analysis. The t(4;11) is associated with as many as 50% of infant ALL, but this drops to 5% in children and adults hence has limited application (Rubin and Rowley 1993). The Philadelphia chromosome has been detected in over 50% of adult and 5% of childhood ALL by molecular probing (Maurer et al 1991), however PCR analysis is an RNA based

technique (Lee et al 1988) and difficult to quantify.

Rearrangements of the Tal1 locus has also been used as a marker of malignancy but is only applicable to a subgroup of patients with T-ALL (Jonsson et al 1991). The low frequency of TcRδ-chain rearrangements in AML limit their use in MRD analysis however the t(15;17) found in virtually every case of acute promyelocytic leukaemia has been amplified by PCR (Castaigne et al 1992). Translocation breakpoints from (11;14) cell lines have also been cloned (Meeker et al 1991) which may have potential application in some ALL.

Of the methods which do not involve molecular techniques, dual colour immunofluorescence has the highest reported sensitivity (van Dongen et al 1992, Campana et al 1990a). This can be applied to a subgroup of patients with B- and T- lineage ALL expressing combinations of cell surface molecules not associated with normal haemopoietic cells. Under these conditions, sensitivity at the 10^{-4} to 10^{-5} level has been reported, though such detection is immunophenotype dependent and fresh material is required for investigation. Campana et al (1990b) compared dual colour immunophentoypic and TcR Vδ1-Jδ1 and Vδ2-Dδ3 PCR analysis in 4 ALL and found that in 2 cases PCR detected residual disease when immunophenotyping could not, whereas immunologically detectable populations accompanied PCR positivity in the other 2 patients. Immunologic methods are also applicable to some AML

expressing cross-compartmental markers, but the presence of such markers on normal cells reduces the sensitivity to approximately 10^{-3}.

FISH has also been successfully applied to MRD analysis in ALL (Anastasi et al 1991) but the sensitivity is not comparable with that of PCR analysis. The predictive value of culturing neoplastic lymphocytes has also been investigated in ALL, although this does not allow the sensitive analysis of disease progression (Estrov et al 1986).

3.6.4 Conclusion

Southern analysis with genomic probes can identify clonally expanded populations of cells, but its relative insensitivity renders it of little value in assessing residual disease. PCR provides detection of low copy number sequences hence can successfully be applied to MRD detection in one of 8 T-ALL and 19 of 36 B-lineage ALL. The junctional diversity displayed at Vδ1-Jδ1 rearrangements in T-ALL provides sensitivity at the level of one cell in 10^5 normal cells. Vδ2-Dδ3 junctional diversity is relatively restricted but the use of oligonucleotide probes can approach a similar level of sensitivity when B-lineage ALL is studied.

CHAPTER IV

IMMUNOGLOBULIN HEAVY CHAIN ANALYSIS

4.1 INTRODUCTION

4.1.1 Role of immunoglobulins

A diverse repertoire of immunoglobulin molecules are synthesised by mature B-lymphocytes enabling the recognition of foreign antigens. Hypervariable regions bind determinants presented by foreign matter leading to ingestion by macrophages or the formation of insoluble aggregates. Recognition of bacterial surface proteins signals the activation of the complement cascade resulting in lysis of the invading cell.

Immunoglobulins consist of 2 heavy and 2 light chains linked by disulphide bonds with the N terminal end interacting with antigen and the C-terminal end determining the class of molecule. The heavy chain is composed of variable (V), diversity (D) and joining (J) segments and the class of molecule is determined by the selection of one of 5 α, γ ε, δ or μ constant region genes. IgM molecules are the first to be produced and use the μ gene although subsequent class switching occurs via the selection of alternative constant regions. Two classes of light chain exist, κ and λ which both have V and J regions, but no D segments. Assembled immunoglobulin molecules use either 2 κ or 2 λ chains, never one of each, a phenomenon termed isotypic

exclusion (Alberts et al 1989).

4.1.2 Generation of antibody diversity

To provide the variation required to recognise foreign particles, both heavy and light chains are assembled from arrays of V, D (in the case of heavy chain) and J segments which are selected at random and joined via a recombinase system similar to that described for TcR genes (Tonegawa 1983). A hierarchy of somatic recombination events analogous to those in TcR genes have been described, with the IgH genes rearranging first followed by κ then λ light chains. The IgH locus on chromosome 14 consists of the 2500kb VH locus containing 6 families with 200 members, which is interspersed with approximately 30 D segments, and the 3kb JH locus comprising 6 J segments. Random selection of segments from these arrays facilitates a large degree of combinatorial variability. Initially one D segment recombines to one J segment mediated via the heptamer/ nonamer RSS before a complete rearrangement is formed by the juxtaposition of a V region to the D-J complex. Diversity is greatly enhanced by junctional variation provided by exonuclease digestion of germline sequences and TdT mediated N nucleotide insertion at the V-D and D-J joins (Alt and Baltimore 1982, Desiderio et al 1984). Affinity of immunoglobulin for antibody is enhanced by somatic hypermutation (Kim et al 1981) via a process called clonal selection. VH segments are divided into hypervariable regions divided by conserved framework

regions which are involved in the assembly of mature molecules. The VNDNJ junction is delineated by framework region 3A and the JH segment, forming the hypervariable complementarity determining region III (CDRIII).

Kappa and lambda light chains undergo a similar rearrangement process, although the germline repertoire is restricted compared to that of the IgH locus. The kappa locus on chromosome 2 consists of at least 100V and 4J segments whilst the lambda genes on chromosome 22 have greater than 6V and 6J segments (Hieter et al 1981).

Heavy chains rearrange at an early stage of B-cell differentiation but a productive, in frame rearrangement may not result, in which case the genes on either chromosome can continue to rearrange. A recombination heptamer (TACTGTG) located at the 3' end of VH segments resembles the signal sequence located immediately 5' to DH segments and lies in the same orientation (Bird et al 1988). This mediates a process termed V-V replacement, whereby one V segment can be replaced by another possibly rescuing an out of frame rearrangement whilst maintaining the DNJ sequence (Wasserman et al 1992a). Once a productive allele is produced, allelic exclusion prevents the second allele from rearranging further and the light chains genes can rearrange, again non-functional genes may result but once a mature gene is assembled a functional antibody can be produced and presented on the cell surface. The cell then undergoes selection in the

bone marrow.

4.1.3 Southern blot analysis

Haematological neoplasms are thought to result from clonal expansions of a single cell hence clonality can be investigated by studying the status of immune receptor genes. Lineage was initially determined using panels of monoclonal antibodies for cell surface lymphoid and myeloid antigens (Foon and Todd 1986). This enabled the differential stage to be characterised, although this was not possible in some immature disorders such as non T- non B- ALL. Southern analysis with IgH probes was employed in the assessment of lineage allowing non T- non B- ALL to be characterised as an early B-cell disorder due to the association of IgH rearrangements with 98% of cases studied (Felix and Poplack 1991). Aisenburg et al (1987) studied 100 cases of B-lineage chronic lymphocytic leukaemia and non-Hodgkin's lymphoma and found that all demonstrated clonal IgH rearrangements, 64% showing a biallelic pattern. This suggests a good correlation between IgH rearrangements and B-lineage but such analysis cannot be used exclusively due to the phenomenon termed "lineage promiscuity" (Greaves et al 1986). IgH rearrangements have been reported in 15% of AML (Adriaanson et al 1991), 10% of T-lineage leukaemia and lymphoma (Kitchingman 1985) and 12% of T-ALL (Felix and Poplack 1991) making unequivocal assignment of lineage impossible on the basis of IgH rearrangements alone.

B-lineage ALL patients have been shown to demonstrate

oligoclonality at the IgH locus in up to 45% of cases at presentation (Beishuizen et al 1991b). This was defined as the appearance of more than 2 rearranged bands in patients shown not to be trisomic for chromosome 14.

4.1.4 PCR amplification of CDRIII sequences in B-lineage disorders

PCR based techniques for the assessment of clonality have several advantages over Southern analysis, in the quantity and quality of material required and in the relative simplicity of the technique.

Yamada et al (1989) first reported the application of PCR to the analysis of IgH rearrangements in B-lineage ALL. This exploited the region of consensus in the FR3A region located at the 3' end of each VH segment and the region of consensus shared by all JH segments, allowing a single pair of primers to amplify 70% of patients. After cloning and sequencing of the PCR products, the junctional diversity was exploited to design clone specific oligonucleotide probes which were hybridised against amplified DNA from post therapy samples. Probing of serial dilutions of tumour DNA indicated sensitivity at the level of one neoplastic cell in 100 000 normal cells.

Trainor et al (1990) reported a PCR based technique for the rapid detection of clonality in B-lineage leukaemia and lymphoma using primers derived from the CDRIII region described above, reporting successful amplification of

junctional sequences in 70% of patients studied. This technique was also appled to paraffin embedded material (Wan et al 1991). Brisco et al (1990) reported a sensitive method of tumour detection which was further refined by the use of sense and anti-sense PCR amplimers designed against unique VND and DNJ sequences respectively from individual patients for the assessment of persisting disease in childhood ALL (Brisco et al 1991).

Modification of PCR products derived from presentation tumour DNA, circumventing the need to synthesise an oligonucleotide probe have been described (Nizet et al 1991). Following CDRIII amplification of presentation DNA using a modified JH primer containing a Sau96I restriction enzyme recognition site, the PCR product was cleaved to excise JH sequences. Chromatography was employed to remove contaminating CDRIII species of a different size and the purified probe radio-labelled extending from a hexamer derived from the 3' end of the VH region to produce a highly patient specific probe. Sensitivity at the 10^4 to 10^5 level was achieved when hybridised to amplified dilution series.

After sequencing CDRIII regions at presentation, Jonsson et al (1990) designed patient specific VND amplimers and used these in conjunction with the conserved JH amplimer. Amplified products were then hybridised against a second patient specific oligonucleotide probe to enhance

specificity and sensitivity, which was reported at the 10^6 level.

However, the use of immunoglobulin specific probes for the assessment of residual disease was thought to be limited by the observation of oligoclonality at presentation and subsequent alteration of banding pattern at relapse (Beishuizen et al 1991a). This could potentially lead to false negative disease assessment if the clonal rearrangement being followed became superseded by a second and suggested that multiple probes may be necessary to track certain patients. However, a number of such bands detected by Southern blotting will derive from incomplete D-J rearrangements (Carter, Neale & Kitchingman 1991) which will not be detected by CDRIII PCR and other changes may derive from V-V replacement or independent V- DJ rearrangement events which will result in the replacement of one V segment with another, yet leaving the D-N-J region unchanged.

Wasserman et al (1992a) described a sensitive system for monitoring oligoclonality at presentation by PCR. Amplified CDRIII products were cloned and 20 independent clones sequenced, sequences were defined as clonal if they were frequently represented or were related to other frequently occurring sequences. Analysis of amplified normal populations indicated that the frequency of any particular rearrangement was one per 20000 lymphocytes hence the likelihood of 2 identical sequences appearing

by chance was very small. Twelve patients were monitored from presentation to relapse with those relapsing within 3 years displaying less clonal evolution i.e. complete clonal change, V-V replacement or V-DJ rearrangements, than those relapsing after 5 years.

Steward et al (1992) reported the use of anti D-N-J probes which will recognise CDRIII sequences, even if a V switch occurs, either by V-V replacement or progressive V-DJ rearrangements. They indicated that complete clonal change in the loss of conserved DNJ sequence was rare and accounted for less than 10% of patients at relapse. This suggested that anti DNJ oligonucleotides could be successfully employed to track disease if care was taken in their design.

The use of a consensus FR3A amplimer does have drawbacks. As the amplimer is derived from a region at the extreme 3' end of the VH gene, exonucleolytic trimming can lead to partial deletion of the binding site, rendering amplification impossible or inefficient. In addition, the degree of mismatch between the primer and particular VH segments may result in poor amplification of some VH family members.

These problems can be circumvented by using family specific VH region FR1 amplimers derived from the 5' end of the gene. These have been used to characterise malignant populations by a technique termed

immunoglobulin "fingerprinting" (Deane and Norton 1991). This involves the use of 6 VH family specific primers in conjunction with a 3' JH primer. Although this technique avoids sequence analysis, it is not as sensitive as those described above but multiple evolving clones can be monitored. Overall, more than 90% of patients with B-lineage disorders can be studied with sensitivity consistently at the 10^3 level. Amplification from FR2 sequences (Ramasamy, Brisco & Morley 1992) and VH family specific leader region primers have also been performed (Campbell et al 1992).

4.16 Aims

I set out to investigate the frequency of IgHJ rearrangements in a range of B-lineage disorders using a Southern blotting technique. A PCR based system was refined and applied to the study of clonal IgH rearrangements. Sequence analysis of a group of PCR positive patients was performed to confirm the origin of the amplified product and used as the basis for the design of patient specific oligonucleotide probes for the sensitive detection of MRD as outlined in the previous chapter.

4.2 PATIENTS

4.2.1 B-lineage ALL

These were a described in section 3.2.2.

4.2.2 Low Grade non-Hodgkin's Lymphoma

These were as described in section 2.2.1.

4.2.3 High Grade non-Hodgkin's Lymphoma

Material from a total of 29 patients with B-lineage high grade non-Hodgkin's lymphoma (18 male, 11 female) with a mean age of 52 years (range 16 to 70 years) was available for analysis.

4.2.4 Hodgkin's lymphoma

Nine patients with HD (8 male, 1 female) with a mean age of 35 years (range 15 to 48 years) were screened.

4.2.5 Myeloma

23 patients with myeloma were available for analysis, 13 male, 10 female, with a mean age of 68 years (range 54 to 84 years).

4.2.6 Cell Line

The Elijah Burkitt's lymphoma cell line was kindly provided by the M.R.C. Human Genetics Unit, Edinburgh.

4.3 MATERIALS

The materials were as detailed in section 3.3.

4.4 METHODS

4.4.1 Restriction endonuclease digestion

This was as detailed in section 2.4.2 except that genomic DNA was digested with EcoRI, HindIII or BglII prior to Southern transfer.

4.4.2 Probe

The IgH joining region probe was as detailed in section 2.4.3.3.

4.4.3 PCR amplification of the IgH CDR III region

This was performed essentially according to Yamada et al (1989). Primers without restriction enzyme sites incorporate at the termini were employed as routine subcloning of PCR amplified DNA was not performed. A consensus FR3 primer (5'- ACACGGC C/T C/G TGTATTACTG -3') was used in conjunction with the consensus JH amplimer described in section 2.4.4. A diagram of the locus showing primer locations is shown in Figure 4.1.

4.4.3.1 PCR conditions

Each reaction contained 0.1μg DNA, 1x PCR buffer, 200nM each dNTP, 30pmol each primer and 1U Taq polymerase. Cycles are detailed in Table 3.3.

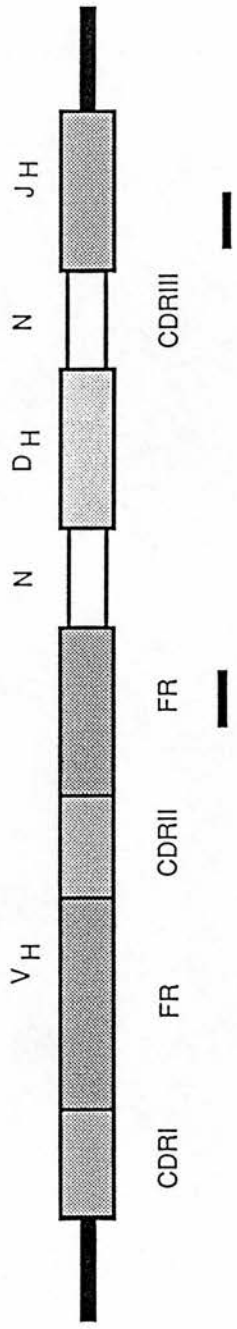


Figure 4.1 Diagram of the IgH CDRIII locus indicating the location of the conserved variable region (VH) and consensus joining region (JH) primers. Also shown are random nucleotide (N) insertions and a Diversity (D) segments. The primers delineate a region of approximately 80 to 120bp.

A magnesium titration was performed using DNA prepared from the Elijah Burkitt's lymphoma cell line in the 0.5 to 3.0mM range, in 0.5mM increments (Figure 4.2). The optimal conditions are seen in lane 4, 1.5mM.

4.4.4 Sequence analysis

4.4.4.1 Purification of PCR products

CDRIII PCR products were excised from ethidium bromide stained 5% PAGE gels with a clean razor blade under UV illumination, crushed and eluted overnight at 37C in 200ml TE pH8.0. The eluates were purified by adhesion to glass beads (MERmaid). Eluates were incubated 5min at RT with 2 volumes of high salt binding solution and 8μl "glass fog". The beads were pelleted at 16000g for 30s and washed twice in ethanol wash solution. Pellets were dried and DNA eluted into 2x10μl dH_2O at 50°C for 5min.

4.4.4.2 Sequencing reaction

This was performed as described in section 2.4.6 with chain extension initiated from either the VH or JH amplimers.

4.4.4.3 Design of anti-junctional oligonucleotides

Probes of 20 nucleotides in length were designed against the junctional region. Generally, these were derived from the DNJ region in order to minimise the effect of VH switching. If little variation was apparent at this site was apparent, then a VND probe was designed. If short junctional sequences were obtained then VN(DN)J probes

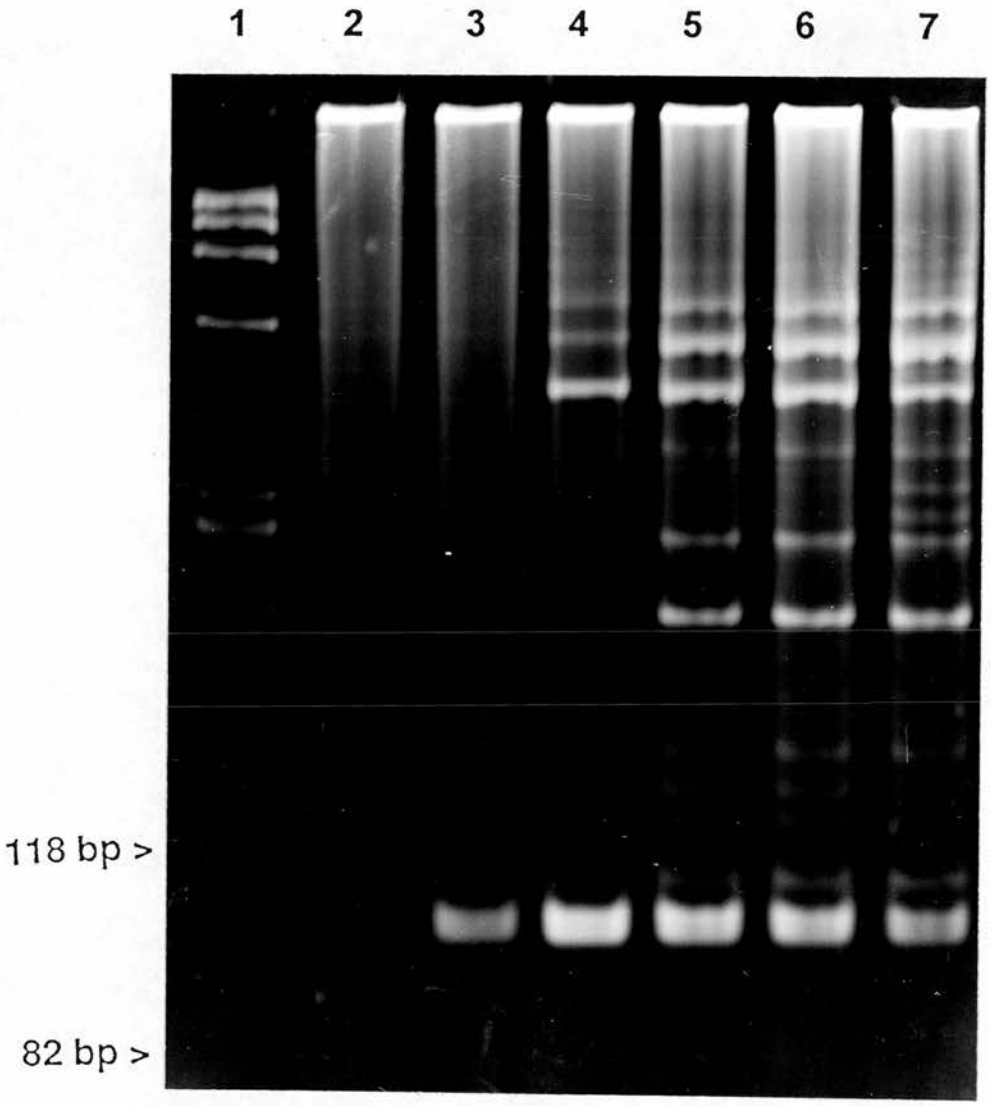


Figure 4.2 Ethidium bromide stained 5%PAGE of PCR amplification products from a Burkitts cell line (Elijah) with a biallelic IgH rearrangement. Lane 1 contains ϕX174 DNA digested with HinfI. Lanes 2 to 7 show the effect on increasing magnesium concentration from 0.5mM (lane 2) to 3mM (lane 7) in 0.5mM increments. The clonal IgH rearrangement can be seen by the presence of a band at 100bp.

were selected.

4.4.5 Determination of residual disease

This was performed according to the rationale and conditions detailed in section 3.4.5.4. Briefly, one microgram of DNA was amplified with the VH and JH primers then 2ml of PCR product slot blotted onto a nylon membrane before hybridisation with the relevant clone specific probe. Bands were visualised by autoradiography after stringent washing.

4.5 RESULTS

4.5.1 Southern blotting

Clonal IgH rearrangements were investigated with the joining region probe described in Chapter 2.

4.5.1.1 Low grade NHL

These were described in section 2.5.1 Of 19 patient samples blotted, all demonstrated rearrangements. Of these 8 were monoallelic and 11 biallelic, results are summarised in Table 4.1.

4.5.1.2 High grade NHL

Of the 12 patients studied by Southern blotting, 10 had detectable rearrangements (83%) with 5 of these (50%) showing a biallelic pattern (Table 4.2).

4.5.1.3 Hodgkin's lymphoma

Patient	Southern	PCR IgH	t(14;18)
SP	RR	+	+
LW	RR	+	+
JC	RR	+	+
JI	RR	ND	+
IT	GR	-	-
WGr	GR	+	+
EB	RR	+	-
JS	GR	+	-
JK	GR	-	-
CL	RR	+	-
AMc	RR	+	-
HG	GR	-	-
WGl	GR	+	-
IM	GR	+	+
AMa	GR	+	+
GN	RR	+	-
AS	RR	ND	-
EW	RR	+	-
JW	RR	-	-
CM	ND	+	+
ML	ND	+	-

Table 4.1 Summary of Southern blot and CDRIII PCR analysis in low grade NHL. G refers to germline configuration and R to rearranged. PCR positivity is indicated by a + symbol, ND indicates not done. t(14;18) results are also included.

Patient	Southern	PCR
MM	RR	+
RB	RR	+
DD	GR	++
DG	ND	-
DE	ND	-
RN	GR	++
VT	GR	-
JH	ND	+
FW	ND	-
IC	GG	-
WH	GR	+
JM	ND	-
IT	ND	+
LM	ND	++
JM	ND	-
MH	ND	+
JE	ND	++
KT	GG	ND
JB	RR	+
DJ	RR	ND
SR	RR	-
SM	ND	++
EI	ND	-
PM	ND	+
WR	ND	+
DM	ND	+++
DP	GR	ND

Table 4.2 Summary of Southern blot and PCR analysis in high grade NHL. G refers to germline configuration and R to rearranged. PCR positivity is indicated by a + symbol. ND indicates not done.

These were as described in section 2.3. Each of 8 patient samples blotted showed germline configuration.

4.5.1.4 Myeloma

Seven of 8 patients tested (75%) had IgH rearrangements (Table 4.3). Of these 6 showed a monoallelic pattern (87.5%) and one 3 non-germline bands.

4.5.1.5 ALL

Twenty two patients with ALL were blotted and of these 18 (82%) had IgH rearrangements (Table 4.4). Seven patients (39%) showed a monoallelic pattern and 7 a biallelic pattern. A further 4 (22%) showed more than 2 rearranged bands. A typical autoradiograph from a blot consisting of a panel of ALL patients is shown in Figure 4.3.

4.5.1.6 Summary

A total of 62 patients with B-lineage disease (this excludes the HD patients) were examined with IgH rearrangements detectable by Southern blot analysis in 53 (85%). Thirty patients had a monoallelic pattern (48%) and 19 had a biallelic pattern (31%) while 5 had 3 or more non-germline bands (8%).

4.5.2 PCR amplification of the IgH CDRIII region

4.5.2.1 Low grade NHL

Fifteen of the 19 patients assayed by PCR displayed a discrete product (79%), each displaying a monoallelic

Patient	Southern	PCR
IW	ND	+
ES	ND	+
MS	ND	+
BC	ND	+
MP	ND	++
LT	ND	+
MW	ND	+
AO	RRR	+++
GC	GR	+
DD	ND	+
JB	GR	-
MF	ND	++
MS	ND	+
FB	ND	-
MF	ND	-
BK	ND	-
ET	ND	-
JW	GG	-
RH	GR	+
MM	GR	-
RM	ND	++
MF	GR	+
WR	GR	ND

Table 4.3 Summary of Southern blot and PCR analysis in Myeloma.G refers to germline configuration and R to rearranged. PCR positivity is indicated by a + symbol. ND indicates not done.

Patient	Southern JH	PCR JH	PCR Vδ2-Dδ3
WL	RRR	+	+
DP	RR	+	+
GG	RR	+	+
RK	GRRR	++	+
BM	GR	+	+
PM	GRRR	+	-
TM	GRRR	+	+
CC	GR	-	-
GH	GR	+	-
FP	GR	-	-
AM	GG	+	-
CAW	RR	-	+
EC	RR	-	+
VH	ND	+	-
LH	GR	+	-
KM	RR	+	-
MS	GG	-	-
HS	RR	++	+
DM	GG	+	-
LW	GG	+	-
MC	ND	++	+
CA	GR	+++	+
DT	ND	++	+
AM	RR	+	-
FW	ND	+	-
DR	GR	+	ND

Table 4.4 Summary of Southern blot and PCR analysis in B-cell ALL. G refers to germline configuration and R to rearranged. PCR positivity is indicated by a + symbol. ND indicates not done. For comparative purposes, the TcRδ-chain PCR results are included.

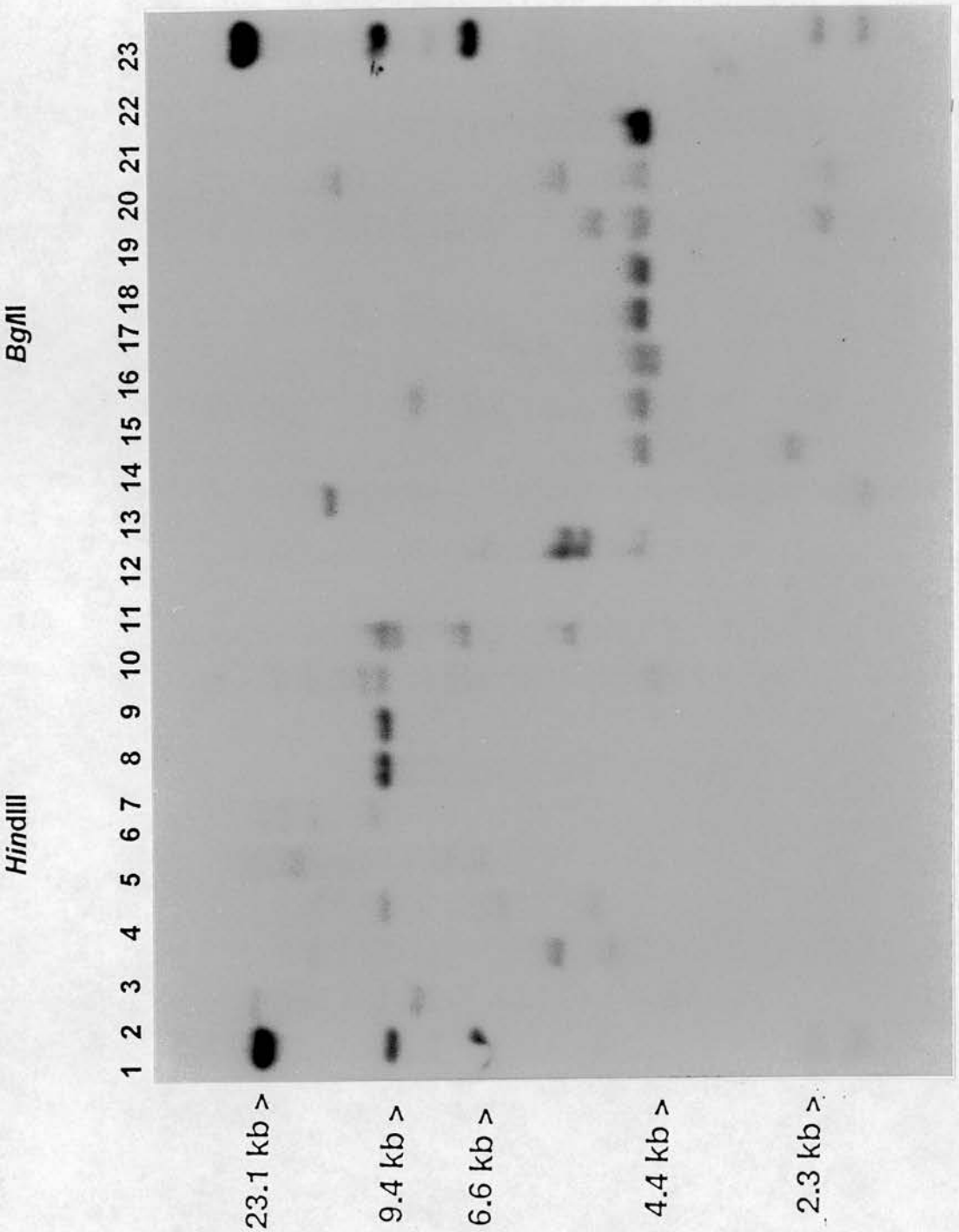


Figure 4.3 Autoradiograph of a Southern blot of 9 patients with ALL. Lanes 1 and 23 contain bacteriophage lambda DNA digested with HindIII. Lanes 2-10 were digested with HindIII and lanes 12-21 with BglII. The blot was hybridised with the IgHJ probe. Germline bands are 9.8kb (HindIII) and 4.5kb (BglII). Lanes 6, 7, 17 and 18 contain DNA from patients with T-ALL with the remainder DNA from patients with cALL.

pattern (Table 4.1). A typical page gel showing the amplification products of a group of B-lineage patients is shown in Figure 4.4.

4.5.2.2 High grade NHL

Of the 24 patients studied, 15 had detectable rearrangements (63%) with 9 of these (60%) showing a monoallelic pattern, 5 a biallelic pattern (33%) and one patient displayed 3 bands (7%) (Table 4.2).

4.5.2.3 Hodgkin's lymphoma

Nine patients with HD were assayed, of these one (11%) displayed an amplification product.

4.5.2.4 Myeloma

Twenty two patients were tested, with 15 showing rearrangements (68%), 11 demonstrating a monoallelic pattern (73%), 2 a biallelic pattern (13%) and one the presence of 3 bands (7%) (Table 4.3).

4.5.2.5 ALL

DNA from 26 patients with ALL was amplified and of these 21 (81%) had IgH rearrangements (Table 4.4). Sixteen patients (76%) showed a monoallelic pattern, 4 a biallelic pattern (19%) and one the presence of three discrete bands (5%).

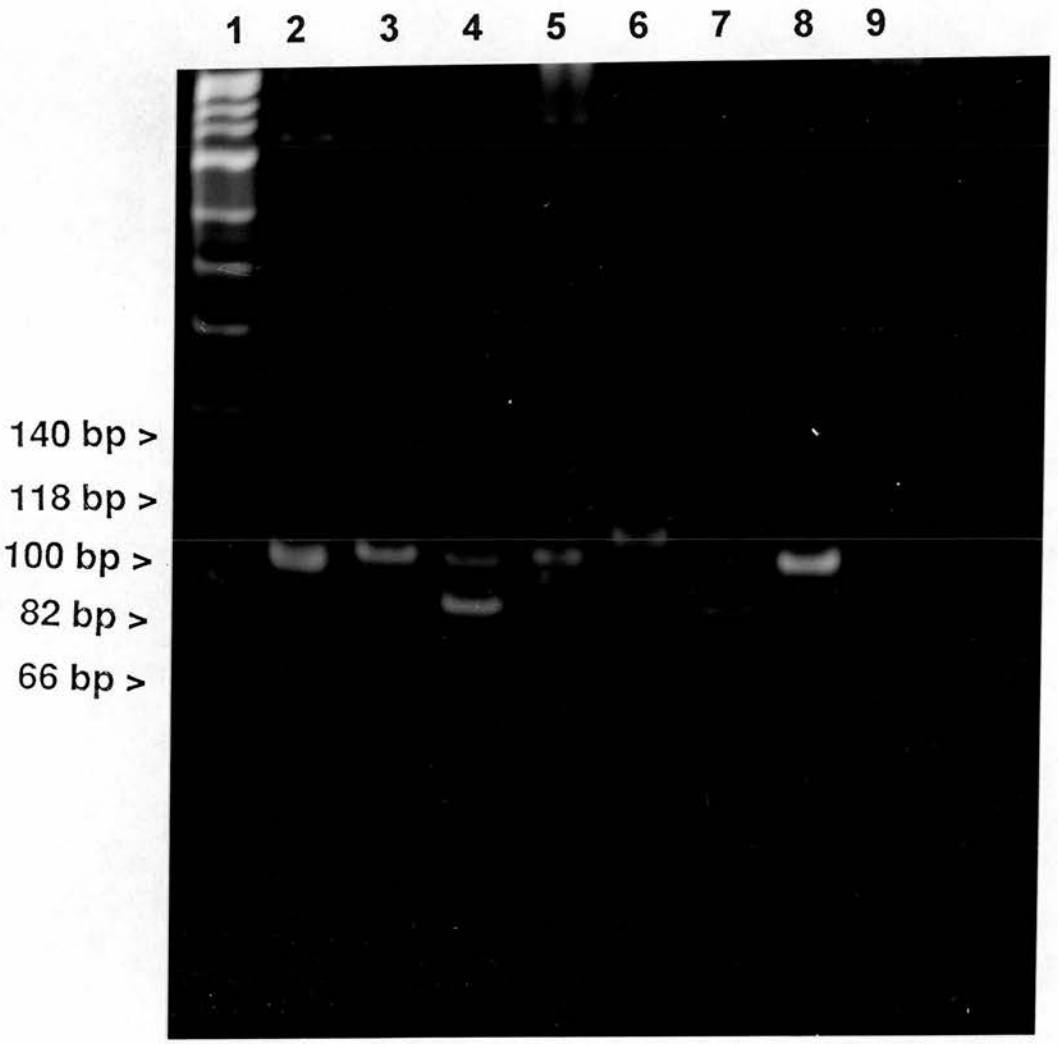


Figure 4.4 Ethidium bromide stained 5% PAGE gel of IgH CDRIII PCR amplification products from 7 patients with B-lineage lymphoma (lanes 2-8) and a normal pooled PBL control. Lane 1 contains ϕX174 digested with HinfI size marker. Band sizes varied from 80 to 120bp. Lanes 2 and 4 contain biallelic rearrangements.

4.5.2.6 Summary

Of 91 patients with B-lineage disease examined by PCR, IgH rearrangements were detected in 70 (77%). Fifty six patients had a monoallelic pattern (80%) and 11 had a biallelic pattern (16%) while 3 had 3 bands (4%).

In total 56 patients were studied with both Southern blotting and PCR with concordance in 40 cases (71.5%). Of these 37 were double positive (66%) and 3 (7%) were double negative. Twelve cases were positive by Southern analysis only (21.5%) and 3 by PCR alone (5.5%). A combination of both techniques with therefore detect clonality in 93% of patients with B-lineage disease (Table 4.5).

Nineteen patients were found to have more than one rearrangement when the data for Southern analysis and PCR was pooled. This excludes the data for the low grade NHL patients as one allele is involved in a translocation event. Only in 2 cases was biclonality detected by PCR when by Southern blotting monoclonality was observed and in one case 3 bands were seen by PCR when only one band was apparent on Southern analysis. In a further one instance biclonality was seen by both techniques. A greater number of rearrangements were observed by Southern blotting than PCR in 15 cases, of these 9 showed one more band by Southern blotting and in the remaining 6 cases at least 2 further rearrangements were detected. This data suggests that PCR will detect fewer IgH

	SB + PCR +	SB- PCR-	SB + PCR -	SB - PCR +	Total
Low NHL	13	-	4	-	17
High NHL	4	1	2	-	7
Myeloma	6	2	2	-	10
ALL	14	1	4	3	22
Total	37	4	12	3	56
%	66	7	21.5	5.5	100

Table 4.5 Comparison of PCR and Southern blotting results. A + symbol indicates detection of a clonal rearrangement whereas a - symbol indicates a failure to do so.

rearrangements than Southern blotting and is relatively poor at detecting biallelism and oligoclonality.

4.5.3 Sequence analysis

In order to confirm the origin of bands resolved on PAGE, direct sequencing was performed on a group of patients. A typical autoradiograph of a sequencing gel is shown in Figure 4.5. Table 4.6 summarises sequence data collected on 13 samples, showing the VH, DH and JH germline sequences along with random N nucleotide insertions.

A diverse range of junctional sequences varying from 12 to 53 nucleotides was obtained. However selective usage of 5' JH segments is found, with J1 and J2 not represented. Biased DH selection can be seen, almost half of the D segments involve either DLR4 or DXP4. Extensive N insertion is common, ranging from 0 to 15 bases, however 4 D-J joins with no random nucleotides are apparent. Further diversity is provided by the selection of multiple DH segments, although in some cases the involvement of no DH segments is found. Deletion of 0 to 12 nucleotides was apparent at the JH terminus, however such extensive deletion at the VH terminus would remove the 5' primer site, hence not be detected.

4.5.4 Analysis of residual disease

4.5.4.1 Design of oligonucleotide probes

Twenty nucleotide probes were synthesised for 7 patients and the Elijah cell line and designed to exploit the

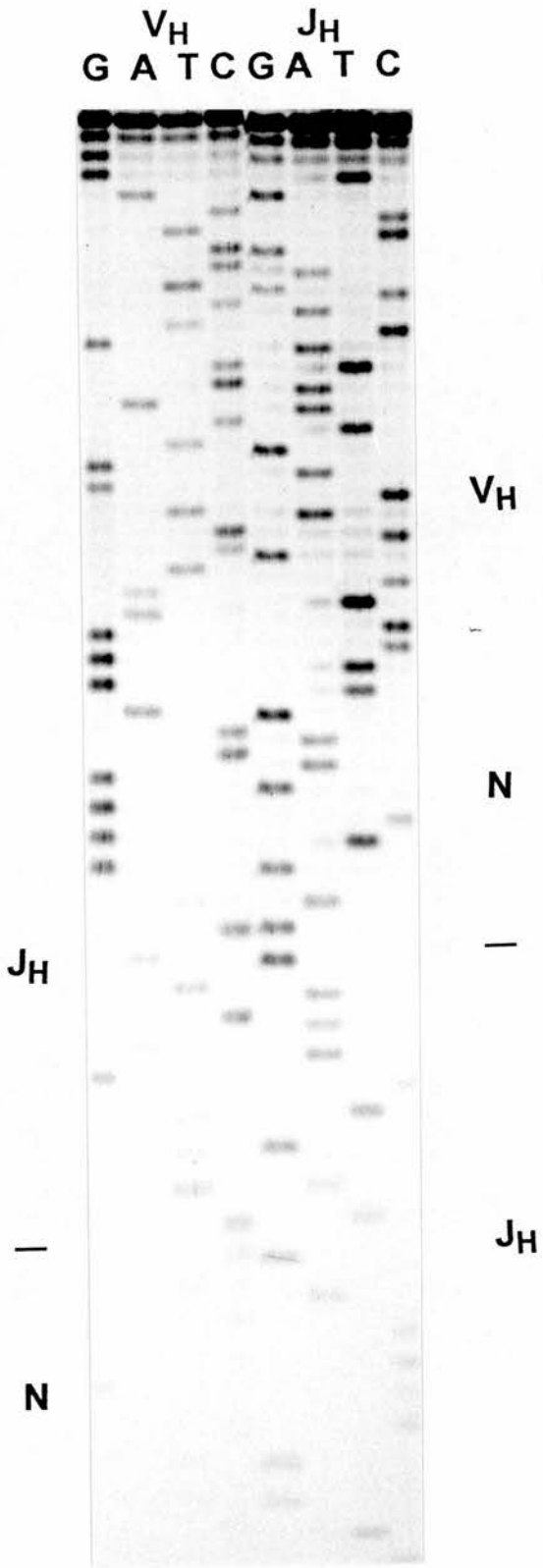


Figure 4.5 Autoradiograph of a 6% denaturing PAGE gel for sequencing PCR products from a high grade NHL patient with an IgH rearrangement. Products were sequenced in both directions using the VH amplimer (left) and JH amplimer (right). Termination reactions were loaded in the order G, A, T and C.

	VH	NDN	JH
AMp	GCGAGA	**CCTTCGTGAACCTGT**	**CTACGG**(J6)
DTr	GCAAGAG	GAGGTGTATGCATTTAT DLR1	TACTAC(J6)
AMr	GCGAGAGA	GGGTGCTAATAATTACGATTTTTGGAGTGGTTATTACCCTA DXP4	TACTACT(J6)
DTr	GCAAGAG	CATGATATTGTAGTAGTACCAGCTGCCCGGGCCGATATTTTGACTGGTTATTA	TACTAC(J6)
DLR4 DXP1			
DTp	GCAAGAGA	TGTTGAGGGTTATTGTAGTAGTACCAGCTGCTAT DLR4	AACCTG(J5)
DTp	GCGAGAGA	GAGCTACGTATTACGATATTTTGACTGGTTAT DXP4	AACTGG(J5)
SM	GCGA	CAAATCTAAG**TGGGGA**ATTTCCTT DHQ52	**GACTTC**(J5)
RB	GCGAGAGAG	GAAGCAGTG**GCT**AACCTTGTGAGAT DN1B	**ACTG**GG(J5)
MM	GCGCGAG	GCAATACGTATTACGATATTTTGACTGG**TTATTATCCGACC** DXP1	**TACTTT**(J4)
JM	**GCGAG**	**GAACTTCGACTC**	**CTTTG**A(J4)
JH	**GCGAGA**	**CATAATGCCAGCGATAG**TAGTCGAACCTGGGGGGC D21/9 DHQ52	TGACTC(J3)
Elijah	**CGAGAGA**	**AGGAGCTAGGAGCAGTGGCTGGTGAT** DLR4	**GCTTTT**(J3)
CL	**G**	**TGGAAAAGCACATCAACGATTTTCGGAAAGG** DXP4	TACTAC(J3)

Table 4.6 CDRIII sequence data. D segments are underlined and named. Probe sequences are indicated in bold and J segment usage indicated in parentheses. Two patients show sequences obtained at presentation (p) and relapse (r).

variation provided by the combinatorial diversity of subunit selection, germline deletion and random nucleotide insertion (indicated in bold, Table 4.6). If sufficient diversity was exhibited at the D-N-J junction, then probes from this region were preferentially selected to minimise the effect of VH switching. However in patients JH and CL a V-N-D probe was designed to the enormous variation within this region, compared to the D-N-J junction. A V-N-J probe was synthesised in the case of AM and JM due to the size of the junctional regions. D-N-J probes were used to analyse MM, SM and RB. Both a V-N-D and a D-N-J oligonucleotide probe was designed against the Elijah junction to compare sensitivity.

4.5.4.2 Sensitivity

Ten fold dilutions of the Elijah Burkitt's lymphoma cell line were made in HL 60 cells, before extraction and amplification of the DNA. The resulting amplification products were slot blotted and probed with either a V-N-D or D-N-J oligonucleotide washed and sensitivity assessed by autoradiography. PAGE resolution of the amplification products is illustrated in Figure 4.6. An autoradiograph of slot blots of the same products hybridised with the VND probe is shown in Figure 4.7. Specific hybridisation can be seen diminishing from the 10^{-1} (lane 1) to 10^{-5} level (lane 5) when probed with the VND sequence, however the DNJ region probe was tenfold less sensitive.

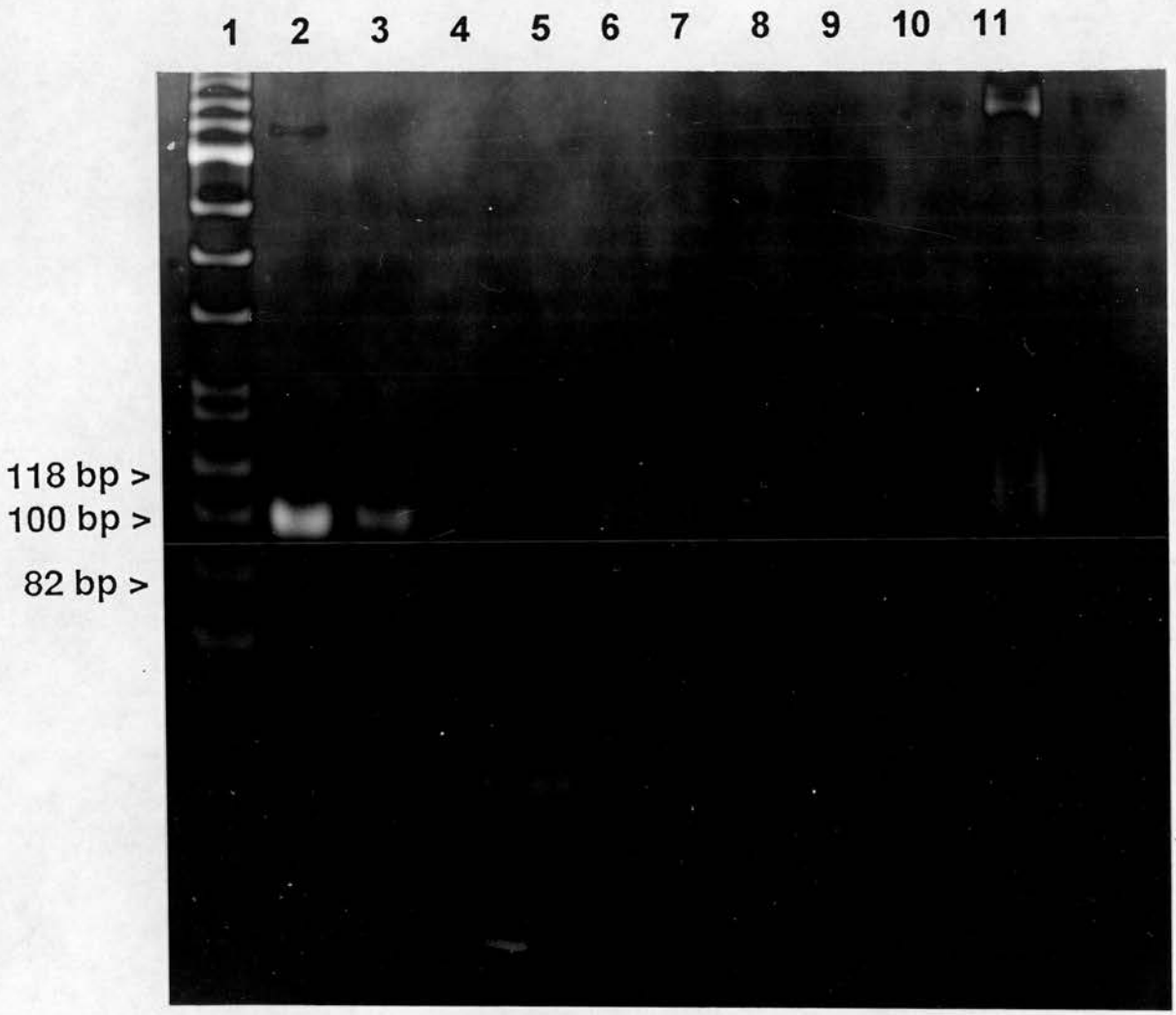


Figure 4.6 Ethidium bromide stained 5% PAGE gel of IgH CDRIII PCR amplification products from a Burkitts cell line (Elijah). Lane 1 contains a ϕX174 digested with HinfI size marker. Tenfold serial dilutions (10^{-1} to 10^{-6}, lanes 3-8) of cell line DNA were amplified. Bands of 100bp indicate the presence of clonal rearrangements. Lane 2 contains undiluted cell line DNA and lane 9 no DNA. Lanes 10 and 11 contain normal PBL DNA.

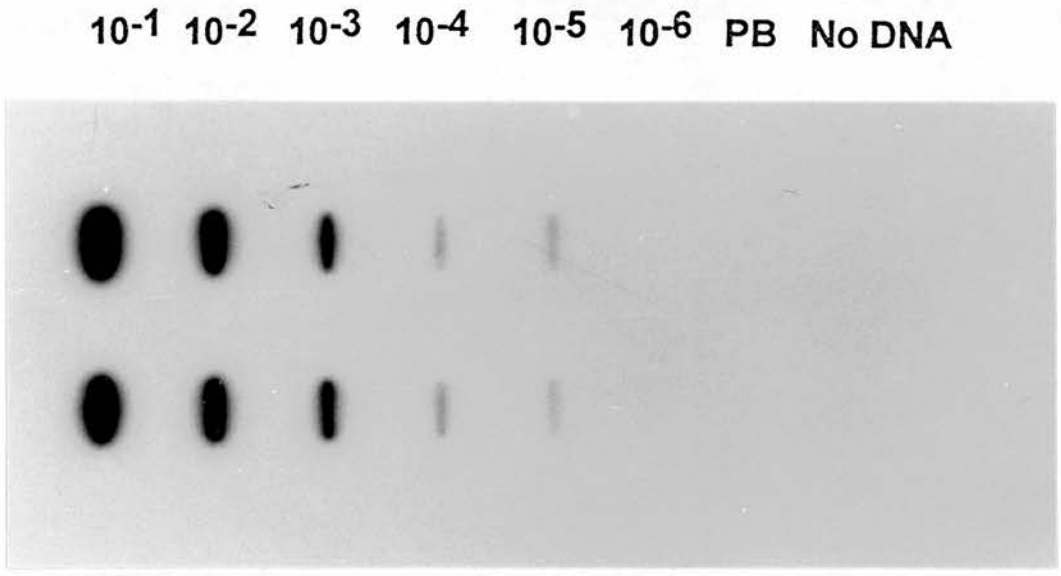


Figure 4.7 Autoradiograph of IgH CDRIII amplification products of serial tenfold dilutions of Elijah DNA in normal PBL DNA (lanes 1-6) hybridised with the VND region probe. Lane one contains the initial dilution point, 10^{-1}, lane 7 contains control PBL DNA and lane 8 contains a no DNA template control. Reactions were performed in duplicate.

4.6 DISCUSSION

Southern blot analysis was used to assess the incidence of clonal IgHJ rearrangements in tumour DNA isolated from patients with a range of B-lineage disorders. Rearrangements were detected in only 83% of patients with high grade NHL, lower than the expected incidence of 100% (Williams et al 1987). Mature lymphoid cells will have undergone rearrangement of one or both heavy chain genes hence the discrepancy may reflect the nature of the biopsy specimens available for analysis, which were not subject to histological examination prior to DNA extraction. The 2 samples found to be in germline configuration may not therefore have been uniformly infiltrated with neoplastic lymphocytes. B-lineage ALL is a disorder of immature lymphocytes therefore a proportion of patients with B-lineage ALL will have IgH genes in germline configuration, thought the incidence here of 82% compared to the previously reported figure of 98% (Felix and Poplack 1991) may reflect the sample size. One patient with myeloma in whom no rearrangement was found may represent a marrow sample heavily contaminated with peripheral blood. Not surprisingly, no IgH rearrangements were found in the group of 8 HD patients as the reported incidence of rearrangements in this group of patients is approximately 10% (reviewed by Daus et al 1989). Overall, the majority of patients with rearranged IgH genes showed a monoallelic configuration (48%) and slightly fewer biallelic rearrangements (31%). Biclonality was

relatively rare, seen in 8% overall and in 22% of B-lineage ALL, though rates as high as 45% have been reported (Beishuizen et al 1991b). This higher rate probably reflects the ability of Beishuizen's technique to consistently detect small populations of subclones which may exist at the 1-5% level and the use of an alternative probe (van Dongen pers. comm.). Biclonality i.e. the presence of three or more non-germline bands may be a poor prognostic indicator (Kitchingman et al 1986), although this has not been confirmed by other studies (Katz et al 1989). A proportion of apparently biclonal patients in this study may be trisomic for chromosome 14, hence not truly biclonal, however cytogenetic information was not available.

Southern blotting is a laborious process which takes approximately 2 weeks to perform and requires upwards of 5-10μg of high molecular weight DNA. It is not therefore an ideal system for the routine analysis of specimens, especially if only archival material, such as frozen lymph node sections or dried bone marrow slides are available. Fortunately, amplification of the IgH CDRIII locus by PCR can be performed rapidly on both fresh and preserved tissue, however the efficiency of clonal rearrangement detection is reduced compared to Southern blotting.

PCR analysis permitted the study of both archival and fresh material so a larger group of patients than those

amenable to Southern analysis could be investigated and overall clonality was detected in 77% of 91 patients with B-lineage disease, compared to 75% of 21 patients reported by Trainor et al (1991) whereas Southern blotting detected clonal rearrangements in 85% of 62 patients. PCR was also consistently poorer at detecting biallelism and biclonality than Southern analysis. These discrepancies can be explained by several reasons, firstly exonucleolytic trimming of the 3' end of the VH segment can delete all or part of the FR3A primer site, secondly the VH amplimer is a consensus amplimer which may match certain VH segments less well than others resulting in inefficient amplification. Hakim et al (1991) aligned the consensus VH amplimer with 40 VH segments and found that 9 had more than one mismatch, though the conserved 3' sequence should ensure reasonably uniform amplification (Rovera et al 1991). Thirdly CDRIII PCR will not amplify partial D-JH rearrangements which will be detected by Southern analysis (Carter et al 1991). Southern blot and PCR analysis concur in 73% of cases, but PCR could not detect clonality in 21.5% of cases which had rearrangements demonstrable by Southern blotting. Therefore PCR is a useful first line assay for clonality although Southern blotting will enable the detection of clonality in a proportion of PCR negative samples. A combination of techniques demonstrated one or more clonal rearrangements in 93% of patient samples at presentation.

Once it was established that PCR generated CDRIII markers were widely applicable in B-lineage disorders and in one patient with HD, the specificity of these markers for possible MRD analysis was investigated. Sequence analysis of 13 bands from 8 patients and one cell line demonstrated large junctional regions which were greater in size than those observed at the Vδ2-Dδ3 locus, due to the formation of complete IgH VDJ rearrangements. This would suggest that probes derived from the CDRIII locus would be more specific than those derived from the Vδ2-Dδ3 region, however there are several considerations. The large pool of circulating polyclonal lymphocytes may contain populations with IgH rearrangements which resemble the patient specific junctional sequence. Such sequences will contribute to non-specific "background" signals when PCR based MRD detection systems using allele specific probes are used. As circulating lymphocytes with Vδ2-Dδ3 rearrangements are less frequent than those with IgH rearrangements (Hara et al 1991), the former may be a better marker even though the sequence variability is restricted. Another consideration is stability of the marker being analysed and the effect of clonal evolution in disease progression, most notably V-V replacement which substitutes VH segments leaving D-JH junctions intact (Wassermann et al 1992a, Potter et al 1992a). Clonal evolution will be discussed in detail in Chapter V. The single bp mismatch between the JH amplimer and J3 does not apparently lead to the under representation of

rearrangements involving this region as they constitute 3 out of 13 CDRIII regions detected, compared to 3 out of 50 of the series of Steward et al (1992). Jonsson et al (1991) investigated the sensitivity of probes derived from either CDR III sequence in patients with biallelic rearrangements and found them to be equally sensitive, though in some cases one VH segment may match the consensus primer better than the other, resulting in differing sensitivities.

The IgH locus provides a specific and sensitive marker for the detection of residual disease, demonstrated by experiments with a cell line indicating sensitivity at the 10^{-4} to 10^{-5} level. The approach outlined here has advantages over other published systems as the routine use of direct sequencing obviates a subcloning stage. Yamada et al (1989) discussed a quantification system based on subcloning and transformation of PCR products into a bacterial host. The degree of tumour infiltration was assessed by comparing colonies positive for JH sequences to those positive for clone specific sequences. This does provide an absolute number, but is an extremely time consuming procedure and assumes that all sequences amplify equally well as the standard sensitivity titration was performed on a cell line and not on an individual patient basis. However an advantage of this technique is that positive colonies can be sequenced to confirm their relation to the original leukaemic clone.

Brisco (1991) described the use of VND and DNJ patient specific PCR primers, however this is expensive especially if more than one rearrangement is studied and does not take the influence of V-V replacement into account. A nested approach was also taken, which is time consuming and prone to contamination (discussed in Chapter III). Jonsson et al (1991) used a similar approach to that above, with the exception that allele specific PCR was performed with a patient specific primer followed by hybridisation with a second patient specific oligonucleotide. This is provides great specificity but is also shares the disadvantages of the Brisco method. Billadeau (1992) also performed an allele specific PCR to investigate PB contamination of plasma cells in patients with myeloma. PCR reactions were performed with the incorporation of ^{32}P and resolved on PAGE gels before densitometric analysis. This permitted accurate quantification of tumour load but was not used to follow residual disease, hence did not take the influence of evolution into account.

Other groups have avoided sequence analysis, Nizet et al (1991) used an approach similar to that detailed in Chapter III described by Hansen-Hagge et al for the TcRδ locus. CDRIII amplification products obtained at presentation were subjected to high power liquid chromatography to remove contaminating species from polyclonal B-lymphocytes after restriction enzyme

digestion to remove some germline JH sequence. These were then radiolabelled and used as clone specific probes. Sensitivity at the 10^{-4} level was reported, however the sensitivity of this technique cannot be as high as that demonstrated by oligonucleotide probes due to the representation of germline sequence within the probe.

Deane and Norton's "fingerprinting" technique (1991) is useful for monitoring subclone progression and was reported to detect clonality in over 90% of patients. Amplification is performed from a 5' FR1 and 3' consensus primer, removing the problem of FR3A primer site deletion and mismatch. However, 6 VH FR1 primers are required to screen patients at presentation for VH family usage. If subclones using a different family emerge then they will not be detected. This technique also requires the running of sequencing gels with P^{32} labelled PCR products and sensitivity is maximally at the 10^{-3} level, insufficient for the detection of low level disease. As no sequencing is involved, potential subclones may be confused with reactive non-neoplastic proliferations which may lead to an overestimation of the incidence of clonal populations.

The considerations associated with FR1 and FR3 PCR analysis were addressed by Ramasamy, Brisco and Morley (1992) who discussed amplification with FR2 and JH specific amplimers. This was successful in 80% of patients but was not evaluated as a sensitive marker for MRD detection. A significant increase in patients

amenable to analysis compared to FR3 amplification was not demonstrated as although deletion of the primer will no longer occur, mismatches with VH family members and the consensus primer persist.

In conclusion, Southern analysis with an IgH probe is more effective at detecting clonal rearrangements than CDRIII PCR amplification, though the rapidity and low cost of the latter technique make it a useful first line assay. It also provides a unique marker applicable in over 70% of patients with B-lineage disease, capable of sensitive disease detection although care has to be taken with probe design to maximise sensitivity and reduce the influence of clonal evolution.

CHAPTER V

PCR ASSESSMENT OF MINIMAL RESIDUAL DISEASE

5.1 INTRODUCTION

5.1.1 Lymphoma

Disease persistence in patients with low grade NHL has been determined by amplification of the t(14;18) translocation. Circulating tumour cells were investigated in patients undergoing therapy to monitor disease progression. Due to the indolent nature of this disease, aggressive treatment regimes may not significantly reduce tumour load and early reports (Crescenzi et al 1988) confirmed the persistence of PCR detectable disease but these studies were derived from relatively few patients. Studies of larger patient cohorts have not involved extensive longitudinal studies but have confirmed the earlier reports of prolonged PCR positivity. A series of 152 patients with low or intermediate grade disease were screened by Gribben et al (1991a) who detected mbr or mcr rearrangements in 102, of whom all had disease detectable by PCR following aggressive therapy. A larger study by the same group reported mbr or mcr translocations in 125 of 205 patients (Gribben et al 1991b) and contamination was revealed in all 114 marrow harvests collected in remission for autologous transplantation. However, following ex vivo purging with anti B-cell determinant

monoclonal antibodies, 57 (50%) of these harvests became PCR negative. Further to this, relapse following re-engraftment occurred in only 4 patients from the PCR negative group, compared to 26 from those receiving PCR positive harvests, suggesting that reinfused tumour contributed to relapse.

Price et al (1991b) reported the persistence of circulating tumour cells in 6 of 15 patients in long term remissions of 11 to 17 years. It was reasoned therefore that the detection of this translocation was not of prognostic significance, confirmed by Lambrechts et al (1992) who studied 11 patients in complete clinical remission and detected the persistence of tumour cells in 5 up to 47 months after the cessation of treatment. However 3 patients with clinically evident disease in the lymph nodes showed no evidence of lymphoma cells in the BM hence the focal nature of the disease can hinder MRD analysis. Recently, a report of normal PB cells bearing t(14;18) markers has been made (Limpens et al 1992) which has implications for the sensitive detection of such events in low grade NHL in remission.

Tumour persistence therefore is common in low grade lymphoma but the finding of Gribben et al (1991) that reinfused tumour can contribute to relapse has implications for PB derived stem cells if contamination is lower than an equivalent BM harvest.

5.1.2 Leukaemia

Several groups have reported semi-quantitative approaches to the monitoring of disease persistence in B- and T-lineage ALL by analysis of CDRIII, TcRδ- and gamma-chain loci. All techniques discussed below are capable of detecting disease at the level of 10^{-4} to 10^{-6}.

Yamada et al (1990) used CDRIII PCR to investigate a group of 8 children with B-lineage ALL. Of the 6 who remained in remission during the time of the study, PCR positivity persisted for up to 25 months from presentation. An increase in tumour load was detected in one patient 3 months prior to bone marrow relapse. The final patient relapsed in the central nervous system, which was not predicted by following tumour contamination in BM samples.

Five cases of B-lineage ALL were analysed by MacIntyre et al (1990) using both TcR γ and δ analysis. Four patients remained in remission of whom 3, sampled at 1, 34 and 38 months respectively, were PCR negative whereas one patient tested positive at one month but negative in a subsequent 26 month BM sample. The final patient showed residual disease at 12 months and relapsed soon afterwards. The results for the 2 loci agreed with one exception, where a high tumour load detected by TcR γ amplification was not detected by TcRδ due to clonal evolution.

Yokota et al (1991a) studied 27 B- and T-lineage ALL patients, of these 8 tested within the first 6 months were PCR positive by Vδ2-Dδ3 analysis. Samples from 11 patients were investigated at 6 to 41 months from the start of treatment, 6 showed persisting positivity. Samples from 11 patients were amplified post therapy and found to be negative with the exception of one who remained positive some 3.5 years after the end of therapy. Two patients in this series relapsed and PCR positivity was detected 4 months prior to morphological detection in each case.

Nizet et al (1991) reported the common finding of PCR positivity up to 6 months in 16 patients investigated by CDRIII PCR. The incidence of PCR positivity was lower in the 7-15 month period and only 2 patients showed residual disease when sampled after the end of therapy. Two patients showed an increasing tumour load 2 to 3 months prior to relapse.

Five patients with T-ALL were followed by TcRδ amplification by Neale et al (1991), of whom one showed an increase in disease 3 months before relapse. Of the remaining 4, who entered long term remission, no persistent disease was detected.

Biondi et al (1992) analysed 20 BM samples from 17 B- or T- lineage ALL patients by Vδ1-Jδ1 or Vδ2-Dδ3 analysis. Ten patients entered long term complete clinical

remission (of duration 52 to 72 months at the time of publication), 9 of whom tested PCR negative in samples collected between 8 and 30 months though one patient was PCR positive at 17 months but negative by 27. Five patients relapsed before the completion of therapy and of these 3 were consistently PCR positive whilst one showed increasing disease though in the fifth case disease could not be detected in a BM sample taken 16 months before relapse. In 2 patients who suffered a relapse after treatment had ceased, disease could not be detected in BM samples collected 2 and 6 months previously.

These studies reported few samples collected at various stages making definitive judgements regarding tumour progression and prognostic significance difficult, although trends are apparent. Early PCR positivity is a common finding and this can persist into the second year of therapy without heralding a relapse. PCR positivity beyond this time may indicate an eventual relapse as patients in long term remission are generally PCR negative. An increasing tumour burden 2-4 months prior to relapse has been described in several studies. Longitudinal analysis rather than the study of a single time point were more informative, although early prediction of relapse was generally not possible.

Other groups have investigated individual key stages in the assessment of risk. Morphological detection of tumour at the end of induction identifies a high risk group

(Miller 1974) and this was extended by Wassermann et al (1992b) who used CDRIII PCR to study 44 children with B-lineage ALL. By day 28, all BM samples studied were in morphological remission but PCR analysis revealed a range of degrees of residual tumour which were semi-quantified by the method of Yamada et al (1989). A 1 to 4 log reduction in disease was reported and no relapses during the course of therapy was reported in the group with the lowest amount of persisting tumour, though late relapses still occurred. Conversely those with only a 1 log reduction relapsed early in treatment in many cases. However this study could not identify all patients with a higher risk of disease recurrence.

5.1.3 Clonal evolution

A necessary condition for the investigation of tumour natural histories is the availability of stable disease specific markers. Cytogenetic markers have been shown to be consistent from presentation to relapse, hence their study was inferred to provide an accurate means for the assessment of residual disease. However, the heterogeneous nature of translocations outwith the Philadelphia chromosome in CML and the t(14;18) in low grade precludes their routine use in the analysis in many haematological neoplasms. Analysis of the t(14;18) lesion has shown this to be consistent from presentation to relapse (Raffeld et al 1987) although the accumulation of subclones with differing breakpoint sequences has been described (Price et al 1991a).

Immune receptor gene rearrangements are associated with most T and B-lineage disease and sufficiently clone specific to provide a marker for analysis. However as gene rearrangements are a continuing process in malignant lymphocytes (Bunin et al 1990), markers may alter as disease progresses. The behaviour of neoplastic cells throughout therapy is dynamic, hence the existence of variant subclones at presentation may result in the appearance of different major clones at relapse. Beishuizen et al (1991) reported that 40% of patients with B-lineage ALL had oligoclonal IgH rearrangements at presentation as demonstrated by Southern blotting. Further to this many patients demonstrated an altered banding pattern at relapse although only 2 patients with completely unrelated rearrangements at relapse were reported. This study did not take into account partial D-J recombination events that can only be detected by Southern analysis but was used as evidence that CDRIII PCR would not be informative in many cases and that TcRδ rearrangements would provide a more stable marker of disease (Beishuizen et al 1992). Potter et al (1992) and Wassermann et al (1992a) used sequence analysis to investigate the role of V-V replacement events, which result in the substitution of a VH segment with one more 5', accounting for the majority of change at the IgH locus. As these events influence the V-D region, the design of D-J region specific oligonucleotide probes

would allow the detection of V-V replacement events. Potter et al reported that using PCR a complete clonal change was seen in only 2 of 32 children with B-lineage ALL. Independent rearrangements of partial DJ recombinations to V segments in subclones will also maintain the DNJ junction whilst generating clones with variant VND sequences.

The stability of the TcR loci has not been as widely documented and it was assumed to provide a more stable marker due to the lower incidence of oligoclonality at presentation. MacIntyre et al (1990) examined 12 δ-chain and 14 γ-chain alleles from 9 patients and found that only one of each changed at relapse. Beishuizen et al (1992) studied 20 cases and reported that by Southern analysis 30% of patients with TcRδ markers showed an altered pattern at relapse.

5.1.4 Aims

I set out to monitor residual disease in a group of patients with leukaemia, lymphoma, and myeloma using a semi-quantitative method to evaluate the prognostic value of PCR analysis. Determination of tumour contamination in PBSCH was compared to that of BMH to test whether these would provide a source of progenitor cells for autologous transplantation with lower tumour infiltration. The effect of clonal evolution in determining MRD was studied in those patients with both presentation and relapse samples.

5.2 PATIENTS

A total of 21 patients with PCR markers were available for further analysis. Eleven ALL patients were studied (10 B-lineage and 1 T-lineage) of which 8 were male and 3 female with a mean age of 15 years (range 3 to 29 years). Five patients with low grade NHL (2 male, 3 female), mean age 46 years (range 44 to 48 years) and 3 patients with high grade NHL (2 male, 1 female), mean age 56 years (range 55 to 57 years) were studied. One patient each with HD (male, 52 years) and myeloma (male, 56 years) was available for analysis.

Fourteen patients underwent PBSC harvesting (5 B-lineage ALL, 4 low grade NHL, 3 high grade NHL, 1 HD and 1 myeloma).

5.3 MATERIALS

These were as previously described.

5.4 METHODS

These were as previously described with the following additions.

5.4.1 Video densitometry

Videodensitometry was used to provide an estimate of

tumour load in patients undergoing therapy (Billadeau et al 1991). Amplified DNA was hybridised against an internal bcl-2 probe (t(14;18) analysis) or a clone specific probe (for IgH CDRIII or TcRδ) as previously detailed. Following a 2 to 4hr exposure, autoradiographs were analysed by video densitometry and OD values calculated. Log_{10} values were calculated from the OD readings obtained from serial tenfold dilutions of tumour DNA in normal mononuclear cell DNA and plotted as a standard calibration graph of log_{10}(OD) against dilution factor. Log_{10} calculations of OD readings from samples obtained during therapy were used to extrapolate an approximate value of tumour load. A standard dilution series derived from SU DH L6 DNA was used to calibrate tumour load in samples from patients with a t(14;18) but individual patient dilutions were used to calibrate the clone specific reactions. Dilutions and serial samples were amplified and hybridised to probes at the same time to ensure consistency.

All reactions were performed in duplicate to minimise variation between reactions and were found to concur to within one log value in each case. Negative controls consisting of normal pooled blood mononuclear cell DNA and no template DNA were run for every series of amplifications.

Graphs were plotted of median tumour load calculated from duplicate reactions using the Microsoft Graph package.

5.4.2 Control PCR

Control primers which amplified a 260bp segment of the Vδ2 gene were employed to test DNA quality. Reactions contained 1x PCR buffer, 200mM each dNTP, 1.5mM $MgCl_2$, 1U Taq polymerase and 30pmol each of a 5' Vδ2 leader region primer (5'- GTCATGTCAGCCATTGAGTT -3') and a 3' Vδ2 primer (5'- TCTCTCTCTGATGGTGCAAG- 3'). Cycles were as detailed in Table 3.3.

5.4.3 PBSC mobilisation and harvesting

PBSC mobilisation was usually mediated by standard chemotherapy consisting of the UKALL XA in ALL, CHOP for NHL and ChlVPP for HD (Craig et al 1992). G-CSF was administered to mobilise stem cells in one case of myeloma. Harvests were collected on a Fenwal CS3000 or Cobe Spectrum cell harvester during recovery phase of the PB count. A total of 62 PBSCH were collected.

5.5 RESULTS

Samples were collected during therapy on 21 patients with leukaemia, lymphoma and myeloma. The persistence of residual disease was correlated with clinical status to evaluate PCR analysis in predicting clinical outcome. Estimation of tumour contamination in PBSC was also undertaken and compared to that in BM sampled concurrently to determine the extent of persisting tumour

in autologous harvests.

5.5.1 Residual disease on treatment

Clinical manifestation of disease is correlated with PCR status for patients with ALL in Table 5.1 and for lymphoma and myeloma in Table 5.2. Semi-quantitative densitometric analysis was used to plot tumour progression a group of 4 patients (3 ALL, 1 low grade NHL with multiple samples (Figure 5.1 A-D).

Clone specific probes from 10 patients with TcR Vδ2-Dδ3 rearrangements were used to analyse remission PB and BM for up to 2 years following induction therapy for B-lineage ALL. Of the 7 patients achieving remission, 3 became PCR negative (CW at 14d, RK at 5mo and MC at 16mo). At 23 months all 3 remained PCR negative and in complete remission. The remaining 4 who entered remission showed persistant PCR positivity, of these one died after one month from causes other than disease and the remaining 3 ultimately relapsed (DP at 23m, DT at 2m and GG at 43m). Reinduction and stem cell transplantation did not result in loss of PCR positivity, all 3 patients subsequently died. PCR positivity persisted in all 3 patient who did not enter remission, with the exception of patient BM, who was transiently PCR negative at day 7 (Figure 5.2). Graphical analysis of tumour progression in 3 ALL is shown in Figure 5.1 A-C.

Analysis of the t(14;18) mbr translocation or CDRIII

Patient	Clinical Status	PCR Status	Probe
WL	remission/ +1mo died from other causes	+ve during remission	10^{-5}
DP	remission/ +23mo relapsed/ died	+ve during remission/ PBSC +ve/ +BMH +ve	nd
DT	remission/ +2mo relapsed/ +6mo PBSCT/ remission/+7mo relapsed/+8mo died	+ve during remission/ PBSC +ve/ +ve following PBSCT	10^{-4}
BM	remission not achieved/ +3mo died	+21d BM and B -ve/ +28d +ve	10^{-4}
GG	remission/ +4mo BMT/ +14mo relapsed/ +14mo relapsed/ remission/ +40mo relapsed/ +43mo died	+ve during remission/ BMH +ve/ +ve following BMT	nd
RK	remains in remission at 24mo	+ve up to +5mo/ remains -ve at +24mo	10^{-5}
CW	remains in remission at +23mo	+14d BM -ve/ remains -ve at 23mo	nd
MC	remains in remission at +23mo	+ve up to 16mo/ remains -ve at +23mo	nd
CA	remission not achieved/ +10mo MUD transplant/ remission/ +18mo relapsed	+ve during remission following BMT	nd
EC	remission not achieved/ +1mo died	remained +ve	nd
T-ALL			
DL	remission/ died post BMT	BMH +ve	10^{-5}

Table 5.1 Summary of clinical and PCR data from 10 patients with B-lineage ALL and one T-ALL. Amplified samples were hybridised against the relevant clone specific probe. Clinical remission was determined by morphologic and immunophenotypic criteria. The final column indicates clone specific probe sensitivity.

Patient	Clinical Status	PCR Status	Probe
low grade	NHL		
SP	remission/ +17mo PBSCT/ remission achieved/ +6mo relapsed	PBSC -ve/ BM +ve in remission/ BM remained -ve 3mo+ BMT	10^{-5}
LW	partial remission/ +19mo relapsed	PBSC +ve	10^{-5}
AM	remission/ +47mo relapsed/ +57mo partial remission / +59mo BMT/ +60mo died	BM -ve in remission/ PBSC +ve in partial remission	10^{-4}
CL	remission achieved post BMT	3mo+ BMT BM -ve	10^{-5}
high grade	NHL		
MM	remission not achieved/ died +21mo	PBSC +ve/ BM +	nd
SM	partial remission/ +10mo BMT/remission/ relapsed +16mo	PBSC -ve/ BM +ve during partial remission	nd
RB	remission/ relapsed +35mo	+4mo PBSC +ve/ +6mo BM -ve	10^{-4}
myeloma			
JM	remission not achieved	PBSC -ve	nd
HD			
JH	remission/ relapsed +7mo/ +15mo PBSCT/ remission	PBSC -ve/ BM +ve during 1st remission	nd

Table 5.2 Summary of clinical and PCR data from 7 patients with B-lineage lymphoma and 1 each of myeloma and HD. Samples were analysed by either t(14;18) amplification or by hybridisation against the relevant clone specific probe. Clinical remission was determined by morphologic or CT scan analysis.

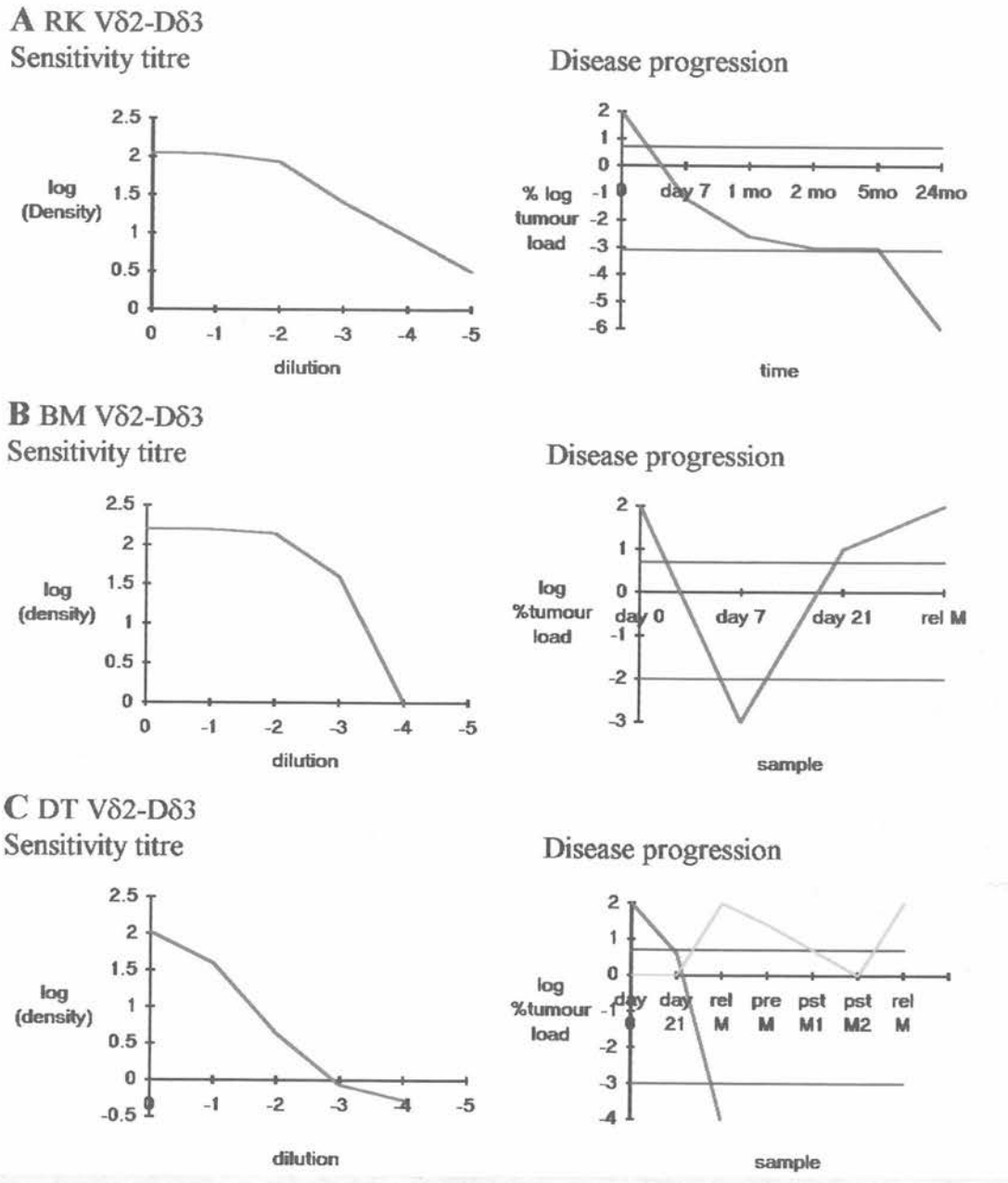


Figure 5.1 Graphical representation of densitometric analysis of sensitivity titrations and disease progression in 3 patients with ALL. The locus studied is indicated after the patients initials. The green line indicates the limit of morphological detection (5%) and the blue line the limit of PCR detection (0.001-0.0001%). In Figure C, the red line depicts clone A (presentation probe) and the yellow line clone B (relapse probe). Rel indicates a relapse specimen, rem remission, pre pre-transplant and pst post-transplant. M indicates a BM sample and mo indicates month.

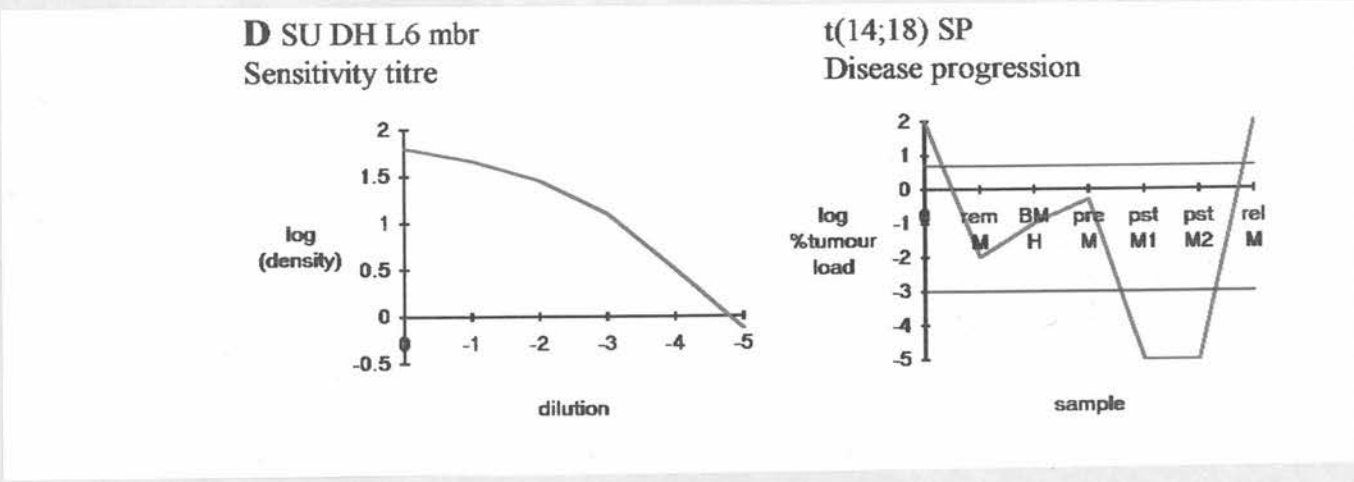


Figure 5.1 continued. Graphical representation of densitometric analysis of sensitivity titrations and disease progression in a patient with low grade NHL. The locus studied is indicated after the patients initials. The green line indicates the limit of morphological detection and the blue line the limit of PCR detection. Rel indicates a relapse specimen, rem remission, pre pre-transplant and pst post-transplant. M indicates a BM sample.

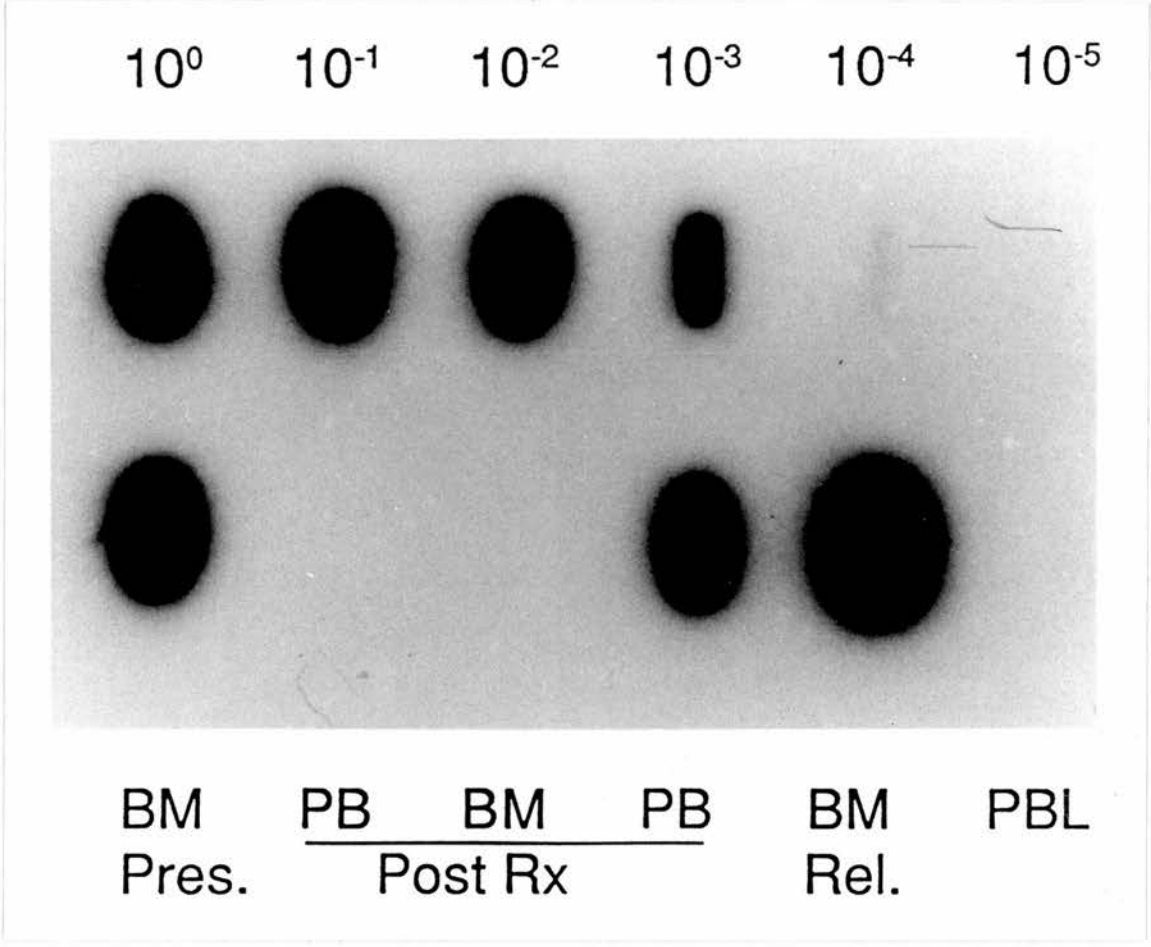


Figure 5.2 Autoradiograph showing Vδ2-Dδ3 amplification products from patient BM hybridised against a clone specific probe. Tenfold serial dilutions of presentation DNA are shown in the top row, lane 1 contains undiluted tumour DNA. Sensitivity is at the 10^{-4} level (lane 5). The lower row contains presentation PB, day 7 PB and BM, day 21 PB relapse BM and normal PBL.

clone specific probes were used to analyse 10 patients with lymphoma and myeloma. Full remission was achieved in 5 patients with lymphoma, 3 showing corresponding PCR negativity (RB, AM and CL) though 2 went on to relapse (RB +33mo and AM +47mo). Of the 2 patients PCR positive in remission, one (SP) became negative after a PBSC transplant (Figure 5.3, densitometric analysis is shown in Figure 5.1D) whilst the other relapsed (JH). Four patients who did not enter full remission remained PCR positive, except the patient with myeloma who had a PCR negative PBSCH.

In patients with lymphoma, dissemination to the BM or PB is not always detectable by morphological means at presentation which has implications for the study of remission BM. Contamination of PB and BM at presentation was consistently detected in either in PB or BM in 7 patients with lymphoma (4 low grade NHL, 3 high grade NHL).

Control PCR reactions were performed on PCR negative samples to exclude the possibility that these were refractory to amplification (Figure 5.4). All samples tested gave a consistent amplification pattern with the exception of the day 7 BM from patient BM.

5.5.2 Tumour contamination in PBSC

A total of 62 PBSCH were collected on 23 occasions from 14 patients and in 8 cases BM samples were collected

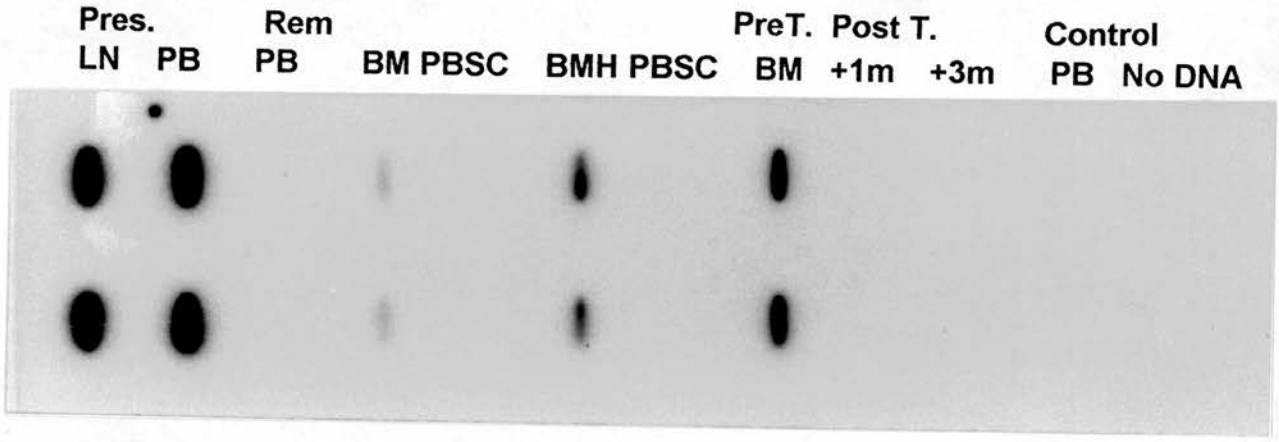


Figure 5.3 Autoradiograph showing t(14;18) mbr amplification products from patient SP hybridised against an internal bcl-2 probe. Pres indicates presentation and rem remission. Pre and Post T. refer to pre- and post-transplant samples. LN denotes lymph node. +1m and +3m indicate the timings of post transplant BM samples.

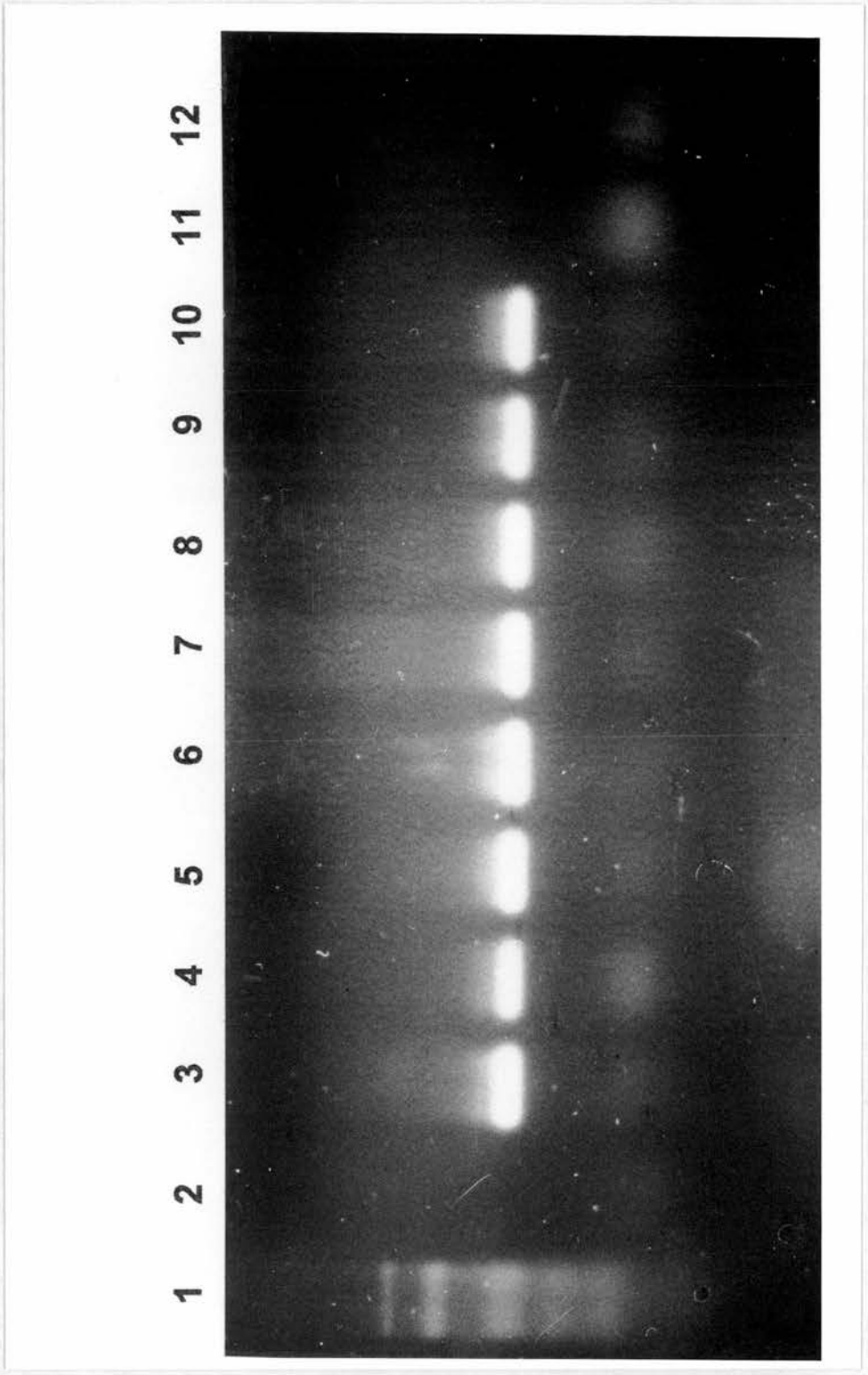


Figure 5.4 Control PCR products resolved by 4% agarose gel electrophoresis and stained in ethidium bromide. Lane 1 contains a ϕX174 size marker. Lanes 2 to 8 contain the following samples: BM day 7 BM; RK +23mo BM; SP PBSCH; SP 3mo + PBSCT BM; SM PBSC; CW +6mo BM and CL 3mo BMT BM. Lanes 9 and 10 contain normal PB DNA and lanes 11 and 12 no DNA controls.

within 3 weeks of the harvest. When Southern analysis with an IgHJ probe was performed on this group of patients (Craig 1991), residual tumour was not detected in any PBSCH and in only one remission BM, indicating that any residual disease present must be at a level of less than 5% (Table 5.3).

The enhanced sensitivity provided by PCR analysis, facilitated the detection of residual disease in 34 harvests from 8 patients including all 5 ALL, 2 low and one high grade NHL. Harvests from 4 ALL collected after successive courses of therapy showed persisting PCR positivity. PCR negative harvests were collected from 6 patients, 2 high grade NHL, 2 low grade NHL, one HD and one myeloma. Figure 5.5 illustrates contamination in both BM and PBSCH collected from a patient with ALL and Figures 5.3 and 5.6 show 2 patients with lymphoma (one high grade and one low high grade respectively) with BM involvement but PCR negative PBSCH. Eight patients had BM sampled within 3 weeks of PBSCH (6 concurrently) 3 of which had residual tumour in BM but not in PBSCH (2 low grade NHL and one high grade NHL). Densitometric analysis of two patients with ALL indicated tumour contamination in the BM was at a level 10 to 100 fold higher than that in the the PBSCH. One ALL, however, had detectable disease in the PBSCH whereas none was detected in a BM sample taken 2 weeks previously. This sample amplified poorly (day 7 BM, patient BM). The results of Southern and PCR analysis are summarised in Table 5.3 with an

Disease	Southern Marker	analysis BM	PBSC	PCR Marker	analysis BM	PBSC
ALL						
GG	JH	G	G	TcRδ	+	+
WL	JH	nd	G	TcRδ	10^{-2}-10^{-3}	10^{-3}-10^{-4}
BM	JH	nd	G	TcRδ	-	10^{-1}
DP	JH	G	G	TcRδ	+	+
DT		nd	nd	TcRδ	10^{-1}	10^{-2}-10^{-3}
high grade	NHL					
RB	JH	R	G	JH	10^{-3}-10^{-4}	-
SM	JH	nd	nd	JH	nd	-
MM	JH	G	G	JH	nd	+
low grade	NHL					
SP	JH	G	G	t(14;18)	10^{-2}-10^{-3}	-
LW	JH	nd	G	t(14;18)	nd	10^{-2}
AM	JH	nd	G	JH	+	-
EP		nd	nd	t(14;18)	nd	+
myeloma						
JM		nd	nd	JH	nd	-
HD						
JH		nd	nd	JH	nd	-

Table 5.3 Comparison of Southern blot and PCR analysis of tumour contamination in PBSCH and BM sampled within 3 weeks. An indication of tumour load is given where quantitation was performed.

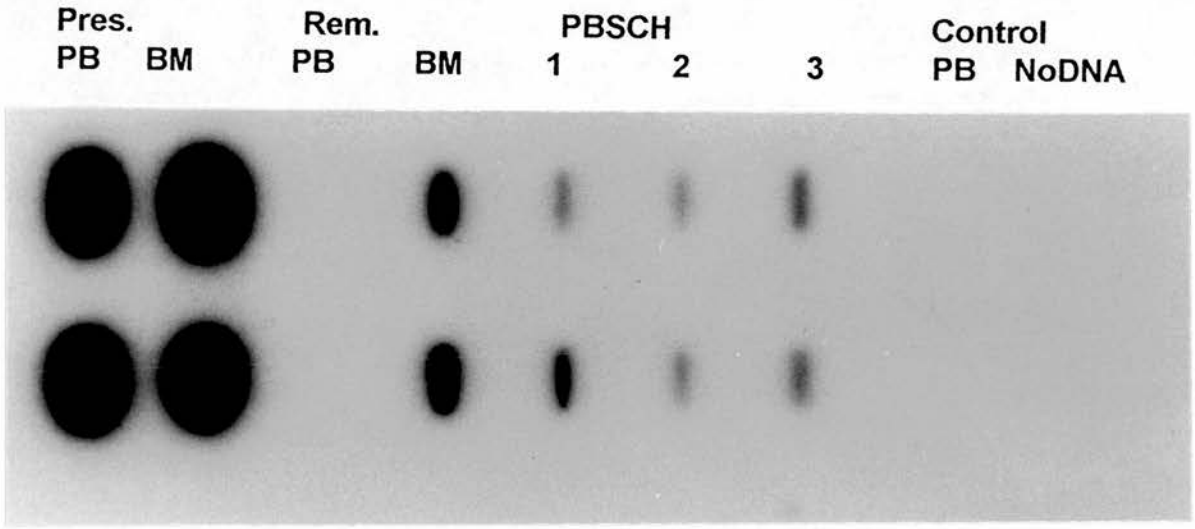


Figure 5.5 Autoradiograph showing Vδ2-Dδ3 amplification products from patient WL hybridised against a clone specific probe. Lanes 1 and 2 contain presentation PB and BM DNA, lanes 3 and 4 remission PB and BM DNA and lanes 5 to 7 PBSCH samples. Lanes 8 and 9 contain control PB and no DNA respectively.

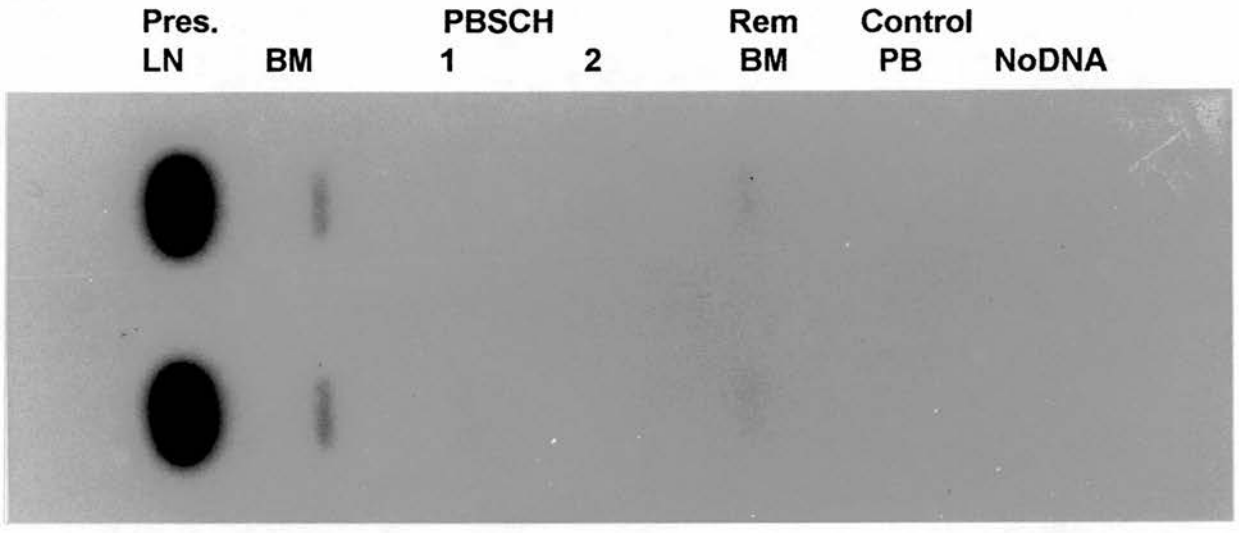


Figure 5.6 Autoradiograph showing CDRIII amplification products from patient SM hybridised against a clone specific probe. Lane 1 contains presentation lymph node DNA and lane 2 presentation BM. Lanes 3 and 4 contain PBSCH samples and lane 5 a remission BM. Lane 6 contains normal PB and lane 7 no DNA.

indication of disease load wherever quantitation was performed.

5.2.3 Clonal evolution

The potential impact of change in clone specific markers was investigated in a group of patients followed to first or second relapse. Vδ2-Dδ3 rearrangements were amplified from 5 B-lineage ALL patients on whom presentation and relapse material was available (including 2 followed to second relapse). One patient (20%) showed a different Vδ2-Dδ3 sequence at first relapse, though this sequence persisted at second relapse. Of the remaining group, all demonstrated consistent junctional sequences, including a second patient followed to second relapse. Material obtained at presentation and relapse was available on 5 patients with low grade NHL, none of whom showed changes in t(14;18) banding pattern at relapse.

A group of 12 patients with B-lineage disorders were analysed from presentation to relapse to investigate evolution at the CDRIII locus. Overall, an alteration in banding pattern was seen in 4 (33%). Sequence analysis in two cases showed the emergence of unrelated sequences which were not due to further V-DJ recombination or V-V replacement events (Table 5.4). In a further 2 cases bands were lost at relapse, one patient initially showed 2 bands, with one present at relapse, the other showed one band at presentation.

Of the 10 patients studied at two loci, change at both

TcR Vδ2-Dδ3 DT

	Vδ2	NDN	Dδ3
Presentation			
	CTGTGCCTGTGACAC	TCCCCATTGT Dδ3	ACTGGGGGATACG
1st/ 2nd	Relapse		
	CTGTGCCT	AGAAGG Dδ2	GGGGGATACG

IgH CDRIII

DT

	VH	DH	JH
Presentation			
	CAAGA	GATGTTGAGGGTTATTGTAGTAGTACCAGCTGCTAT DLR4	AACTGGTT (J5)
	CGAGA	GAGATCTACGTATTACGATATTTTGACTGGTTAT DXP4	AACTGGTT (J5)
1st/ 2nd	Relapse		
	CAAGA	GCATGATATTGTAGTAGTACCAGCTGCCCGGGCCGATATTTTGACTGGTTATTA DLR4 DXP1	TACTACTAC (J6)
	CAAGA	GGAGGTGTATGCATTTAT DLR1	TACTACTAC (J6)

AM

Presentation		
GCGAGA	CCTTCGTGAACCTGT	CTACGG(J6)
Relapse		
GCGAGAGA	GGGTGCTAATAATTACGATTTTTGGAGTGGTTATTACCCTA DXP4	TACTACT(J6)

Table 5.4 Alterations of CDRIII and TcRδ sequences from presentation to relapse. Results of sequence analysis of Vδ2-Dδ3 and IgH CDR III rearrangements from a patient DT with ALL at presentation and first and second relapse are shown. CDR III sequences from a low grade NHL patient AM with from presentation to relapse are also shown. Homology with D segments is indicated with underlining and P nucleotides are shown in bold type.

was only seen in one instance, with change at one locus in 3 cases (Table 5.5).

Changes in specific markers can result in false negative disease assessment but also permit the study of the growth kinetics of distinct sub-clones. Disease progression in a patient with a high risk associated t(4;11) translocation is illustrated in Figure 5.7. A probe sensitive at the 10^{-4} level derived from a Vδ2-Dδ3 sequence at presentation was hybridised against a series of samples collected throughout the course of treatment (Figure 5.7A). Marrow at day 21 was highly contaminated although the patient had entered remission by morphological criteria. However, the clone predominant at presentation could not be detected in the first relapse or subsequently. Sequence analysis of the amplification product obtained at relapse confirmed that the Vδ2-Dδ3 sequence had altered. A second probe specific to the relapse sequence hybridised strongly to the relapse sample, but a signal was also obtained in the original presenting BM hence both clones were initially present (Figure 5.7B). Following PBSC transplantation, the patient again achieved clinical remission, but PCR positivity persisted, even with the stimulation of natural killer cells by IL-2. The clone identified at first relapse was identified at a subsequent second relapse although no trace of the initial clone could be detected suggesting selection for a chemoresistant clone had occurred. The patient died from disease one year from

Patient	t(14;18)	TcRδ	IgH
low NHL			
LW	stable	-	lost 1
SP	stable	-	stable
JI	stable	-	stable
IT	stable	-	stable
IM	stable	-	changed
ALL			
BM	-	stable	stable
DT	-	changed	changed
CA	-	stable	stable
GG	-	stable	stable
DP	-	stable	changed

Table 5.5 Summary of patients with 2 markers analysed presentation to first relapse. A - symbol indicates that this locus was not studied.

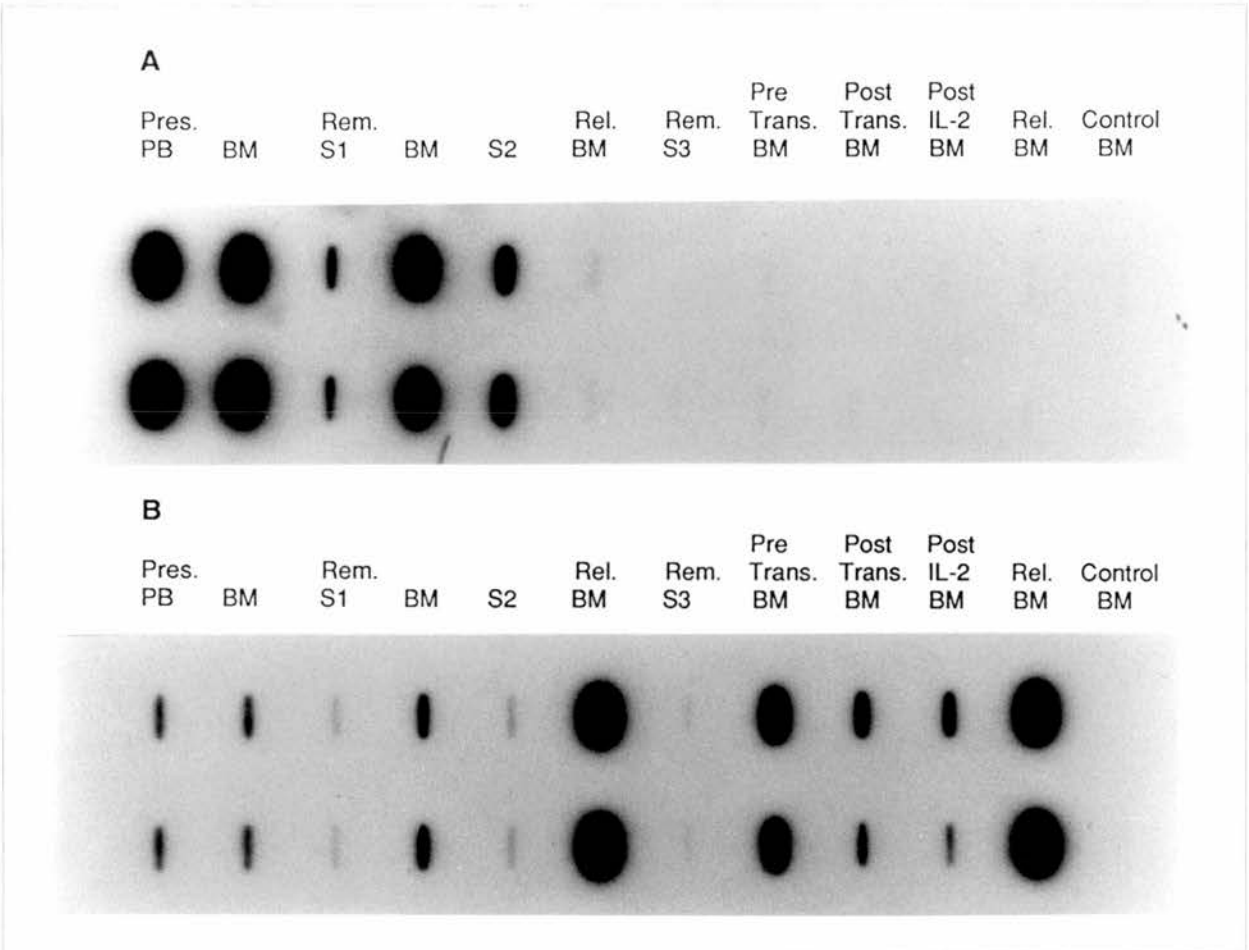


Figure 5.7 Autoradiograph showing Vδ2-Dδ3 amplification products from patient DT hybridised against a clone specific probe. A series of samples collected throughout therapy hybridised against the presentation probe are shown in **A** and the same samples against the relapse probe in **B**. Pres. indicates presentation, rem remission and rel relapse. S1 to S3 indicates PBSCH 1 to 3. Normal BM was used as acontrol in this instance.

presentation. Densitometric data for the two probes is shown in Figure 5.1C. Amplification and sequencing of the IgH CDRIII region also showed an altered pattern at first relapse which remained consistent at second relapse. PAGE analysis of presentation, first relapse and second relapse for both loci is shown in Figure 5.8 and sequence data summarised in Table 5.4.

In summary, analysis of molecular markers can successfully detect tumour contamination in samples previously considered to be in remission. In ALL persisting high level or increasing PCR positivity may indicate relapse whereas those patients in long term remission are generally PCR negative. The focal nature of lymphoma renders relapse prediction more difficult. Blood is generally a poorer indicator of systemic tumour load than marrow although PB derived stem cells did have detectable tumour contamination. Clonal evolution was found to affect 20% and 33% of patients respectively when the TcRδ and CDRIII loci were studied whereas none of the 5 low grade patients showed an altered t(14;18) mbr band.

5.6 DISCUSSION

PCR analysis of tumour specific loci facilitates the study of disease at levels not previously attainable and has redefined the concept of residual disease. The biological significance of sub-microscopic tumour in patients undergoing therapy has been investigated by a

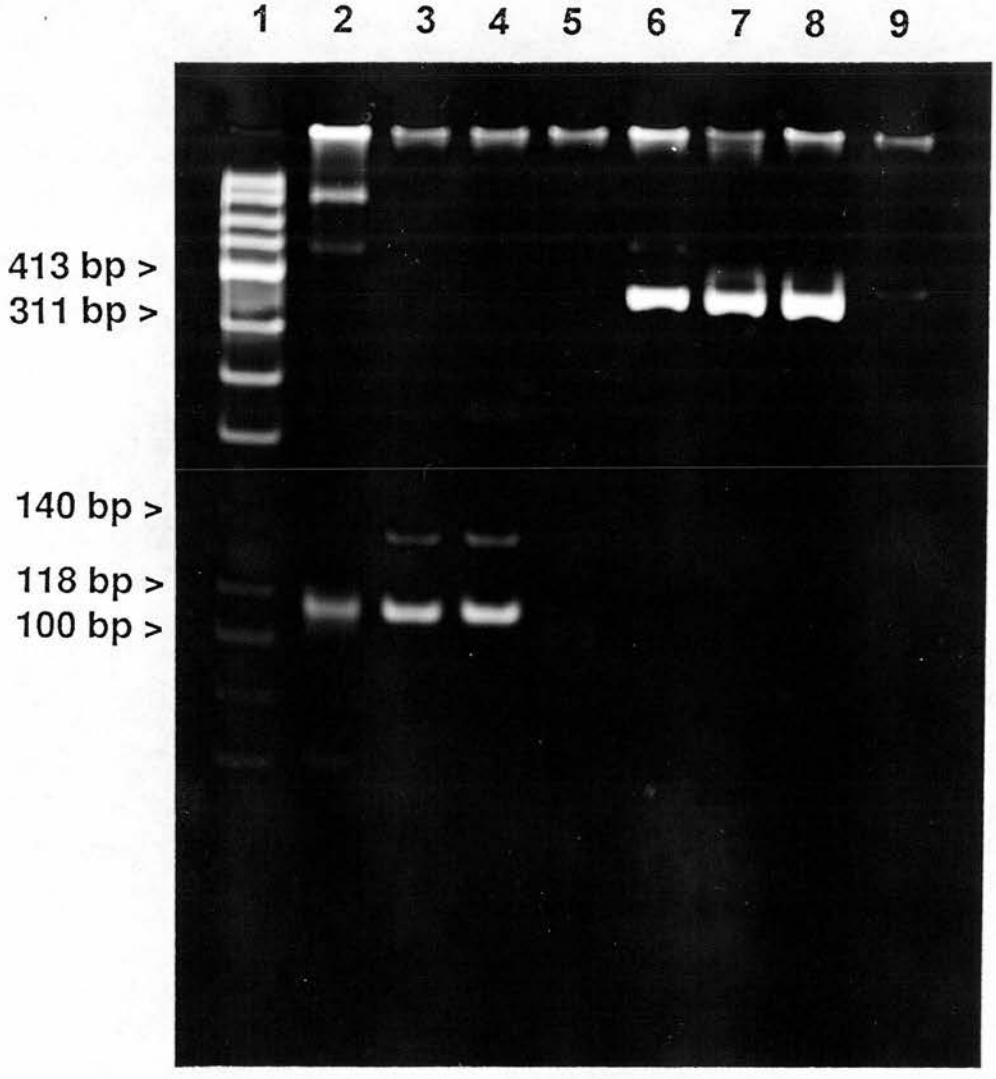


Figure 5.8 PAGE analysis of presentation and relapse PCR products from patient DT stained with ethidium bromide. Lane 1 contains a ϕX174 size marker and lanes 2 to 5 contain presentation, 1st relapse, second relapse and a normal DNA control respectively amplified with the CDRIII primers and lanes 6 to 9 the same samples amplified with the Vδ2-Dδ3 primers

variety of techniques, however definitive conclusions cannot be drawn due to the relatively small numbers of patients studied and heterogeneity of sample collection and differences in treatment protocols.

In this study I have attempted to correlate clinical observations with PCR status in order to evaluate the possible prognostic significance of MRD detection in patients with leukaemia and lymphoma. Further to this tumour infiltration in PBSCH was investigated as it has been suggested that these provide a less contaminated source of progenitor cells than autologous BM harvests reducing the load of reinfused tumour cells which may contribute to relapse.

Trends were apparent when longitudinal analysis was performed in patients with ALL. PCR positivity in the first year of treatment was a common finding hence not predictive of eventual relapse although quantitation of samples collected from 4 patients after induction showed varying degrees of residual tumour. Rapid relapse was observed in 2 patient with a disease load greater than 10^{-3} whereas the remaining 2 showed persisting tumour at a level of less than 10^{-4} and subsequently entered long term remission. Wasserman et al (1992b) studied a larger group and suggested that the level of disease detected following induction could identify patients at risk of early relapse, however patients likely to relapse further out could not be identified. Positivity persisted into

the second year of therapy in 3 cases although relapse occurred only in 2 therefore persisting tumour at this time does not necessarily herald relapse, confirmed by Yamada et al (1990) who reported the persistence of positivity up to 25 months in patients in remission. One patient who achieved a partial remission had a negative result at day 7, followed by a positive result at day 21 and went on to relapse after 3 months. This seemingly anomalous finding was explained by a DNA sample partially refractory to amplification. The 3 patients who achieved long term remission became PCR negative between 14 days and 16 months hence PCR negativity is associated with a better prognosis. Relapses were always preceded by PCR positive samples in this study. Yokota et al (1991) reported a fall in the incidence of PCR positivity with time and they and others (Macintyre et al 1990, Nizet et al 1991 , Neale et al 1991 and Biondi et al 1992) found that patients in long term remission were generally PCR negative. Another common report was an increase in tumour burden in patients prior to relapse, though relapse could only be predicted less than 6 months in beforehand. Only one T-ALL was available for analysis, who showed persisting disease at the time of a BMH and subsequently died post-transplant.

Increasing PCR positivity appears to be an important indicator of relapse, hence quantitative analysis may provide more accurate disease assessment. Various methods have been described including limiting dilutions (Yokota

et al 1990), the phage colony titre assay (Yamada et al 1989), densitometric analysis (Billadeau et al 1991) and simple comparison to standard dilution series (Potter et al 1992) to determine tumour specific TcRδ and CDRIII template concentrations. The basic problems with quantitation stem from the logarithmic nature of PCR, varying amplification efficiencies due to variation in conditions such as enzyme activity and the requirement for representative dilutions. For these reasons all such methods assume accuracy to within one log, therefore a simple estimation of tumour load by comparison to individual patient dilution curves should provide a meaningful estimate of tumour load. The method used in this study was less exhaustive than the densitometric method described by Billadeau et al (1991) as an absolute figure for tumour contamination was not calculated, rather a useful means to represent tumour progression. Dilutions appear to provide linear titres in the sub 10^{-3} range, the level at which residual disease may be of clinical relevance hence provide accurate values for tumour load in this range. More exhaustive techniques, such as the phage colony titre assay which compares the tumour contamination detected by CDRIII specific probes to a cell line bearing an IgH rearrangement diluted in normal cells, does not take into account the differential amplification of individual patient sequences hence will not necessarily provide a more accurate indication of tumour load than the method described in this study. However the phage titre technique permits the sequence

analysis of clones testing positive, a useful check against non-specific hybridisation of CDRIII probes.

Fewer sample points were collected in patients outwith the ALL group. The persistence of PCR positivity into clinical remission was seen in 2 of 5 patients. Of the 3 patients with no detectable disease 2 went on to relapse, in one case only 3 months after a PCR negative sample. In this series therefore relapse prediction wasn't possible. Full remission was not achieved in 4 patients and PCR positivity persisted in all these patients with one exception. A PCR negative PBSCH was collected from the patient with myeloma even when BM involvement was clinically evident. Billadeau et al (1992) studied PB involvement in untreated patients with myeloma and found levels of disease unrelated to that observed in BM which may explain this finding. It would appear that MRD analysis is of most value in ALL, due to the nature of samples available. Lymph nodes cannot be routinely biopsied and PB or BM sampling do not appear to provide an indication of systemic tumour load. As low grade NHL is characterised by slowly growing cells, the persistence of disease does not indicate a poor prognosis (Price et al 1991b), nor does the reduction of disease indicate cure.

Disease progression in patients undergoing allogeneic transplantation is modulated by donor T-cell activation

which can result in a potentially lethal GVHD and associated, though beneficial, GVL effect (Goldman et al 1988). Tumour persistence following allogeneic transplantation has been studied in a large cohort of patients with CML. Miyamura et al (1992) reported the frequent detection of the bcr-abl transcript in patients achieving sustained clinical remissions of durations of up to 3 years at the time of publication. This did not necessarily herald the onset of relapse although patients who became PCR negative did not relapse. Cross et al (1993) analysed 61 patients who underwent allogeneic transplantation in first chronic phase for CML and reported that patients generally remained PCR positive for periods up to 9 months with positivity beyond this time identifying a group at risk of relapse, although this was not predictive on an individual patient basis. All relapses were preceded by a positive result, but relapses could occur only one year after a PCR negative result. A correlation between chronic GVHD and PCR negativity was not found and this was taken as evidence of a separate graft mediated anti-leukaemia effect. Quantitation of RNA based PCR is difficult but competitive inhibition experiments may provide an idea of tumour load and be of further prognostic value. A murine model simulating high risk ALL undergoing transplantation from a sibling donor has been developed (Drobyski, Baxter-Lowe and Truitt 1993), in which male donor cells were detected in a female recipient by Y specific PCR This may facilitate the study of GVHD and GVL effects in

vitro.

Tumour infused in an autologous graft may contribute to relapse as well as endogenous disease surviving the conditioning regime. In addition to this no donor T-cell mediated GVL effect arises, leading to higher relapse rates than that seen with allografting. Purging has been performed in an attempt to reduce contaminating tumour and PCR analysis allows the effectiveness of this procedure to be monitored. Gribben et al (1991a) employed immunologic purging of BMH in 114 low grade NHL with anti CD10 and CD20 monoclonal antibodies. Amplification of the t(14;18) translocation indicated that all harvests were initially contaminated but purging resulted in a 3 to 6 log reduction in tumour load with 57 becoming PCR negative. Post-transplant, the majority of relapses came from the group receiving PCR positive harvests suggesting that infused disease can contribute to relapse. Retroviral tagging with a neomycin resistance gene has been used to investigate the source of relapse in 2 patients undergoing autologous transplantation for AML (Brenner et al 1993). Marked cells from the harvest were detected in both cases at relapse which followed a period of PCR negativity concomitant with remission state hence it was apparent that disease introduced along with BMH could contribute to relapse.

These findings suggest that methods minimising reinfused tumour could improve the outcome of autografting and such

an improvement may be provided by PBSC transplantation. It was assumed that stem cells derived from the peripheral circulation would provide a source of autologous stem cells less contaminated by tumour (To et al 1987) although there has been little experimental evidence to confirm this. Bell et al (1990) used a Southern blot technique to detect tumour in BM at a time when PBSC were apparently tumour free. Yokota et al (1991) observed that concurrently sampled PB from ALL patients in remission was 10 to 100 fold less contaminated with persisting disease than BMH however no data exists on the detection of tumour contamination in PBSC harvests by PCR techniques.

Of the 13 patients analysed, tumour was detected in PB harvests from 8, hence these still harboured a significant tumour load. Of the 5 in whom no tumour could be detected, 3 showed contamination in a concurrently sampled bone marrow. In 2 patients with contaminated BM and PBSC, semi-quantitative analysis indicated that the BM contained an approximate tenfold higher tumour burden. This indicates that in 5 cases where both PBSC and BM were analysed, PBSC would have provided a less contaminated source of stem cells. In only one case did a BM sample show lower disease contamination than a PBSCH. The leukapheresis collection procedure enriches for the mononuclear cell fraction which contains the neoplastic lymphocytes, possibly increasing tumour load. Further to this, approximately tenfold more cells are reinfused in a

PBSC transplant as compared to a BM transplant, hence a similar level of contaminating tumour cells may be introduced with both techniques, although the increase in accessory cells with PBSC enhances engraftment and may modulate disease progression. Three patients underwent PBSC transplants, of whom 2 received cells containing no discernible disease. SP showed residual disease in all BM sampled prior to transplantation whereas post-transplant BM samples were PCR negative, hence a reduction in circulating disease was achieved although the patient relapsed six months post-transplant. Patient JH received PCR negative stem cells before a subsequent relapse. In the third instance patient DT received harvests contaminated by 2 clones characterised by discrete TcRδ and CDRIII markers of which the clone identified at second relapse was apparently chemoresistant. Only this clone was evident at second relapse although a significant amount of the chemosensitive presenting clone was reinfused with the PBSCH. As the relapse occurred shortly after transplantation, it suggests that endogenous cells surviving myeloablative therapy brought about relapse rather than reinfused tumour. This would explain the presence of the second clone, if the graft was responsible for relapse then both clones would have been represented. It would appear in this patient that an earlier cell with IgH and TcR genes in germline configuration was producing daughter cells which had different rearrangements as the clones observed at relapse could not be explained by further recombination

of the genes seen at presentation. The persistence of a t(4;11) during the course of disease confirms the common origin of the clones. This simulates the "tagging" model outlined by Brenner et al (1993), hence the study of patients undergoing evolution may provide an alternative to retroviral mediated marking. A leukaemic clone with evidence for a progenitor with stem cell features was also reported by Tycko et al (1992). They reported a malignant clone with both CDRIII and TcR γ rearrangements which maintained CDRIII sequences at relapse, accompanied by a <u>de novo</u> V γ sequence.

The response of a larger number of patients infused with PBSC assayed by PCR are required before the role of reinfused tumour can be determined. Enrichment for stem cells, expressing markers such as CD34 may provide the least contaminated source of autologous cells (Civin et l 1990), although the removal of more mature accessory cells may hinder engraftment. An anti leukaemic effect is stimulated by the action of IL-2 on natural killer cells (reviewed Hamblin 1988), though this is only effective when disease bulk is significantly reduced. IL-2 was administered to DT post PBSCH, but tumour detectable by PCR may represent too high a level of residual disease for successful immune modulation of tumour.

The differences in disease behaviour observed between leukaemia and lymphoma highlights one of the problems associated with MRD detection irrespective of the

sensitivity of the technique employed, that is the focal nature of disease. Leukaemic cells may not be disseminated in the peripheral circulation, likewise tumour may be localised in certain niches within the BM which may not be sampled when marrow is collected (Martens et al 1987). Such BM specimens can also be contaminated with PB at the time of collection which could potentially reduce the sensitivity of the assay by 10 to 100 fold. The focal nature of lymphoma is a greater problem for the analysis of residual disease as routine collection of lymph node biopsies is not practical and disease may not be disseminated to the peripheral circulation even when significant nodal infiltration can be detected histologically (Lambrechts et al 1992). In 7 patients analysed at presentation, disseminated disease was detected in each instance though this may not necessarily provide an accurate indication of systemic tumour load during therapy. Problems of residual disease analysis in HD are increased by the relative rarity of Reed-Sternberg cells within lymph nodes which may exist in even lower levels PB or BM.

There is also a finite limit to the quantity of BM that can be biopsied and amount of DNA that can be analysed. Maximally 10μg of DNA can be amplified giving an optimal sensitivity of 1 tumour cell in a background of 10^6 normal cells and even with this level of detection 10^5 occult neoplastic cells can persist although realistically, 1μg provides a better substrate for

amplification. This in conjunction with the rapid doubling time of a malignant clone could lead to relapse soon after a PCR negative sample as has been obtained. Sensitive detection of low level disease gives no indication as to the clonogenic potential of the cell population detected. Residual tumour contamination in CML may originate in terminal cells transcribing bcr-abl message which have become incapable of proliferation, likewise leukaemic lymphoblasts may differentiate and lose the ability to divide whilst maintaining TcR or CDRIII marker sequences. Further to this PCR may amplify DNA fragments released from cells lysed by chemotherapy, hence sampling soon after therapy may overestimate residual tumour.

Another major consideration is the stability of the marker under analysis. It is crucial that the locus chosen to follow disease progression remains stable if prospective investigation of the significance of MRD is to be meaningful. Beishuizen et al (1992) estimated the evolution rate at the TcRδ chain locus by Southern blot analysis to be 30%, and of 5 patients examined at relapse in this study one showed change. This was a subtle alteration which would not change the pattern observed on Southern blot analysis, hence Beishuizen's figure may be an underestimate. This patient showed a consistent pattern at second relapse, hence the synthesis of a single new probe allowed analysis of all intermediate samples. This is not a problem for retrospective analysis

however steps must be taken to minimise the effect of clonal change if prospective studies are to be undertaken. Of the 10 patients followed to second relapse, 4 showed an alteration in CDRIII banding pattern, although in only 2 was this pattern unrelated to that seen at presentation. In contradiction to earlier reports, the IgH CDRIII locus may provide a very stable marker if care is taken in probe design. Potter et al (1992) reported on a series of 30 patients from presentation to relapse, the major source of clonal change was found to be due to V-V replacement events. If DNJ region probes were designed, only 6% of patients would lose a detectable marker at relapse. The rates of biallelism and oligoclonality at presentation are higher at the IgH than TcRδ locus (39 and 6% respectively in our series), hence a greater number of probes would have to be synthesised if each IgH allele was to be followed. A total of 87 CDRIII probes for 70 patients with leukaemia, lymphoma and myeloma, an average of 1.24 probes per patient would be required to follow each rearranged allele, though this figure is an overestimate as DNJ probes will detect related subclones in many cases. Jonsson et al (1991) investigated the relative sensitivities of probes derived from either allele in a cell line with 2 rearrangements and found equivalent sensitivities. Potter et al 1992 calculated that 1.5 probes per patient would be adequate to follow all patients with B-lineage ALL. Of the patients studied by CDRIII analysis in this study, only one (patient SM) was

biallelic, though disease progression was only followed with a probe specific to one rearranged allele.

The design of CDRIII DNJ probes is difficult if little diversity is displayed at this junction. If 2 loci are studied, a probe derived from the most diverse sequence, either CDRIII or TcRδ would be best selected. In this study 10 of 25 patients with B-lineage ALL (40%) shared IgH and Vδ2-Dδ3 rearrangements. Vδ2-Dδ3 positive ALL patients represent a selected subgroup, however a larger group could be investigated if 2 or more loci are studied. In one patient (DT) with B-lineage ALL, both the Vδ2-Dδ3 and IgH rearrangements were amplified and sequenced but as both sequences changed from presentation to relapse, probe design considerations would have been ineffective due to the effect of complete clonal change. If all patients with B-lineage ALL were followed by both TcRδ and CDRIII analysis, a total of 38 probes in 25 patients would be required, an average of 1.8 per patient. The t(14;18) translocation in low grade NHL provides an extremely stable and tumour specific marker though analysis of CDRIII rearrangements does facilitate the study of a larger group of patients.

PCR analysis of multiple loci is an exhaustive process but the use of multiplex PCR to screen a variety of markers simultaneously may both complement cytogenetic analysis and identify patients amenable to MRD investigation. Izraeli et al 1993 co-amplified 4 loci

covering each of 2 variants of t(1;19) and t(9;22) rearrangements in precursor B-ALL simplifying the process of analysing a large number of patients for relatively infrequent markers. This allowed the detection of these lesions in 11% of 121 patients, the structure of which could then be analysed by both single strand conformation polymorphism (SSCP) (Kovar et al 1991) and sequence analysis.

The sensitivity of PCR analysis may be improved by the enrichment of biopsied marrow for B-lymphocytes. Bregni et al (1989) reported a tenfold enhancement in the sensitivity of Southern analysis when myeloid cells were removed by incubating with monoclonal antibodies. Treatment of BM with B-cell specific markers such as CD10 or CD20 may also provide such an increase in sensitivity when extracted DNA is analysed by PCR, which will further characterise the disease state in ALL and may enhance the prognostic significance of MRD analysis in lymphoma.

The recent development of <u>in situ</u> PCR (Nuovo et al 1991) allows the detection of leukaemia specific signals in intact cells, enabling amplification of morphological slide preparations. Sensitivity is limited by the number of cells which can be analysed, but the technology would be applicable to the analysis of cultured cells or sorted cell populations. This would involve amplification of rearranged loci with at least one CDRIII or TcRδ junction specific primer to provide specificity. Amplification

products are then hybridised to an internal digoxygenin labelled oligonucleotide probe followed by chemiluminescent detection. This would be of particular interest in determining the nature of lymphoid blasts observed by light microscopy and may allow clearer definition of remission state or in the analysis of lymph node biopsies.

An alternative to immune gene PCR could be provided by "vectorette" PCR (Arnold & Hodgson 1991) which enables discrete amplification with single primer specificity via the use of synthetic linkers. This involves the digestion of genomic DNA and ligation of vectorette linkers to create libraries which are then screened by PCR. Many translocations have not been sequenced or have heterogeneous breakpoints and require an RNA template for amplification due to the variation in size of the breakpoint region. This gives no size discrimination of products between patients hence carry-over is a potential problem. Translocations in which one side of the breakpoint is known are amenable to vectorette PCR such as the t(9;22) bcr translocation in CML and ALL (Mills et al 1992). Primers specific to the vectorette and to exon b3 of the M-bcr were used to amplify and sequence the junctional sequence from 3 patients with CML. This allowed the design of patient specific PCR primers allowing DNA based PCR. In addition to removing the need for RNA this also reduces the problem of carry over and eases quantification. Such an approach would facilitate

the amplification and sequencing of translocations involving TcR or IgH loci from other haematological disorders providing stable targets for MRD analysis which do not have to be differentiated from polyclonal sequences as with immune gene markers.

The prognostic value of PCR analysis can only be improved on by the implementation of prospective coordinated multi-centre trials. Two such trials are currently underway, the first involves the study of residual disease in T and B lineage ALL in children being treated on the Berlin Frankfurt Munster (BFM) treatment protocol in Germany, Holland and Italy (Potter et al 1993 in press). In marrow sampled at 3 month intervals over a 5 year period, MRD will be assessed by analysis of TcRδ, TcRgamma and Tal-1. A second trial, involving children with ALL on the UKALL X/XI treatment protocol is currently underway in Britain to investigate disease persisting at the end of therapy and possible prognostic significance (Steward et al 1993). In this trial, duplicate samples will studied by TcRδ, TcRγ , t(9;22) and IgH CDRI and III analysis different participating centres providing corroboration of results and checks against false positivity.

In conclusion, it is apparent that the implementation of sensitive techniques has refined the definition of MRD. Such techniques have been of particular interest in the study of leukaemia, whereby marrow sampling can provide

an indication of systemic disease. The results of several studies show that the incidence of PCR positivity falls with the increase in remission duration and relapse is generally preceded by a PCR positive sample. PCR analysis appears to be less informative in the study of lymphoma due to the localisation of disease within the lymph nodes. PCR analysis has shown that harvests collected from PB are not necessarily free from tumour but they do appear to represent a less contaminated source of progenitor cells, which purging studies suggest may reduce the risk of relapse associated with autologous transplantation.

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APPENDICES

Publications and Presentations

Publications

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Presentations

Langlands, K., Craig, J.I.O., Parker, A.C. & Anthony, R.S. Molecular determination of minimal residual disease in peripheral blood stem cell harvests. Bone Marrow Transplantation 1990 **5** (suppl. 1) 64-65. Oral presentation to the First International Symposium On Peripheral Blood Stem Cell Transplantation, Mulhouse, France, October 1989

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Langlands, K., Parker, A.C. & Anthony RS. TcRδ sequence analysis: applicability to the monotoring of MRD in cALL. Oral presentation to the 4th workshop of the molecular biology/ BMT study group, Bristol, May 1992

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Langlands, K., Parker, A.C. & Anthony, R.S. Analysis of TcRδ rearrangements in common acute lymphoblastic leukaemia. Oral presentation to the British Society Of Immunology Autumn Meeting, London, November, 1992

Langlands K., Craig J. I. O., Parker A. C. & Anthony R. S. Molecular Determination of Tumour Contamination in Peripheral Blood Stem Cell Harvest. Experimental Haematology 1990 **18** 680. Poster presentation to the International Society for Experimental Haematology, XIX Annual Meeting, Seattle USA, August 1990.

Langlands, K., Craig, J. I. O., Parker, A.C. & Anthony R. S. Analysis of minimal residual disease in peripheral blood stem cell harvests. Poster presentation to European Bone Marrow Transplant Group Meeting, Cortina, January 1991.

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Direct sequence analysis of TCR Vδ2–Dδ3 rearrangements in common acute lymphoblastic leukaemia and application to detection of minimal residual disease

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Summary. T cell receptor δ chain (TCRδ) gene rearrangements were studied by Southern blot analysis in 36 patients with common acute lymphoblastic leukaemia, including 14 adults and 22 children. The majority of patients (68%) had either a rearrangement or deletion of one or more TCRδ genes. The most frequent rearrangement involved a partial recombination of Vδ2 to Dδ3 (55%). Dδ2–Dδ3 rearrangements were present in five patients (14%). To investigate the TCRδ rearrangement as a tumour marker in minimal residual disease studies, presentation samples from 18 patients were amplified by PCR and directly sequenced. Although the size of the Vδ2–Dδ3 junction varied by only 40 bp, sequence analysis showed extensive diversity. This was derived from four factors: deletion of the 5′ end of Dδ3 gene (15/18) and 3′ end of Vδ2 gene (16/18); the presence of Dδ2 sequences (6/18); insertion of N nucleotides (15/18); association of P nucleotides with intact Vδ2 and Dδ3 genes (5/18). N nucleotides were the major feature, contributing to 75% of the junction. Dδ1 sequences were not involved. Twenty base oligonucleotide probes, constructed from the junctional sequences, were capable of detecting residual tumour cells at the 10^{-4} sensitivity level. Cross hybridization studies confirmed the probes to be clone specific. Longitudinal studies on patients undergoing treatment were capable of detecting tumour in remission samples.

A major clinical problem in the treatment of patients with leukaemia is the presence of residual neoplastic cells following induction remission therapy. Improved evaluation of minimal residual disease would assist in monitoring an individual patient's response to therapy and provide an early indication of impending relapse. Analysis of recombination events associated with immune receptor genes in B- and T-lineage disorders is of use in monitoring clonal populations of neoplastic cells. Immunoglobulin heavy chain (IgH) gene and T-cell receptor (TCR) gene rearrangements were previously assayed by the classical methods of Southern blotting and gene probing but these were limited by poor sensitivity. More recently, the application of techniques based on enzymatic amplification of immune receptor genes by polymerase chain reaction (PCR) has identified minimal residual populations of tumour cells. One such PCR based strategy involves the rearrangement of the TCRδ chain gene. Two heterodimer forms of TCR are described, termed αβ and γδ (Allison *et al.* 1982; Brenner *et al.* 1986). Lymphocytes which express γδ are present as a minor population (1–10%) in the peripheral blood (Lanier & Weiss, 1986). Although TCR molecules are expressed by T-lymphocytes, rearrangements of TCR genes appear not to be lineage restricted, most notably, TCRδ rearrangements have been reported in 70% (Hara *et al.* 1988) and TCRβ rearrangements in 30% of B-cell precursor acute lymphoblastic leukaemia (ALL) (Hara *et al.* 1989).

Diverse mature TCRδ chains result from the rearrangement of variable (V), diversity (D) and joining (J) gene segments during the early stages of T-cell differentiation. The number of functional V, D and J segments is limited in TCRδ genes (six, three and three respectively), however the junctional diversity is determined by the presence of D gene segments, deletion of germline nucleotides and randomly inserted N nucleotides (Hata *et al.* 1988; Loh *et al.* 1987). The limited germline diversity encoded at the TCRδ locus (Takihara *et al.* 1989) in conjunction with extensive junctional diversity results in lymphocyte clones which provide a useful target for PCR analysis. The restricted Vδ2(D)Dδ3 configu-

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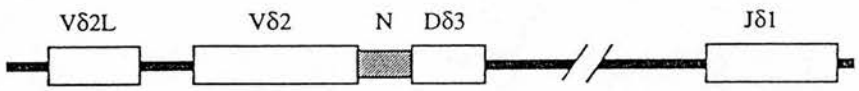

PCR primers

sequencing primer

PRIMER SEQUENCES

PCR primers	Vδ2	5' - GAG TCA TGT CAG CCA TTG AG - 3'
	Dδ3	5' - AGG GAA ATG GCA CTT TTG CC - 3'
sequencing primer	Vδ2	5' - GCA CCA TCA GAG AGA GAT GA - 3'

Fig 1. Genomic organization of the partial TCR Vδ2–Dδ3 rearrangement showing the locations and sequences of primers for PCR amplification of Vδ2–Dδ3 and of the sequencing primer.

ration, frequently associated with common ALL (cALL) (Biondi *et al.* 1990), can be exploited to generate oligonucleotide probes specific for individual clones and used to monitor minimal residual disease (Yokota *et al.* 1991b). Sequence analysis of these junctions can be performed to investigate the mechanisms involved in recombination and also has implications in the study of T-cell ontogeny (Yokota *et al.* 1991a).

We aimed to study a group of adults and children with cALL for the presence of TCR Vδ2–Dδ3 gene rearrangements both by Southern blot analysis and by PCR amplification. In 18 of these patients we further analysed the Vδ2–Dδ3 rearrangement by direct sequence analysis of the PCR reaction product and from the sequence data we were able to construct clone specific probes for use in assessing minimal residual disease.

METHODS

Cell samples. Bone marrow or peripheral blood samples were obtained at presentation from 36 patients with cALL (MIC criteria, First MIC Cooperative Study Group, 1986). These included 14 adults with median age 33 years and range 16–60 years, and 22 children, median age 6 years and range 2–14 years. Normal peripheral blood mononuclear cells were separated by Ficoll density gradient centrifugation. High molecular weight DNA was prepared by phenol/chloroform extraction following lysis and proteinase K digestion as described (Sambrook *et al.* 1989). 7 μg of genomic DNA was digested to completion with restriction endonucleases HindIII or BglII, electrophoresed through 0·8% agarose and transferred to nylon membranes. Membranes were hybridized at 65°C in 7% sodium dodecyl sulphate (SDS), 0·25 M NaH_2PO_4 with a $J\delta_1$ probe (JδS16 probe; Boehm *et al.* 1988). The probe was radio labelled by multipriming (Feinberg & Vogelstein, 1983) to a specific activity greater than 5×10^8 cpm/μg DNA. Membranes were exposed to X-ray film after stringent washing.

PCR analysis. PCR analysis was essentially performed as described by Saiki *et al* (1988). Each 100 μl reaction mixture contained 10 mM Tris (pH 8·3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM each dNTP, 1 U Taq polymerase (Advanced Biotechnologies, U.K.) and 30 pmol of each primer (Fig 1) (Yokota *et al.* 1991b). Either 100 ng of DNA from presentation samples of 1 μg of DNA from post-treatment samples was included per reaction. The PCR reaction mixture was incubated for 35 cycles at 94°C for 90 s, 55°C for 60 s and 72°C for 90 s. In the final cycle, the extension time was increased to 7 min at 72°C. Products were analysed by 5% polyacrylamide gel electrophoresis and visualized with ethidium bromide staining. Sample cross-contamination was avoided by processing and storing DNA in a separate location from PCR analysis and by using aerosol tips and fresh solutions for each patient.

Direct sequencing analysis. The PCR reaction products from presentation or relapse samples were purified using glass milk (Geneclean, Stratech Scientific, U.K.) and sequenced using the modified method of Winship (1989) together with Sequenase (USB, Cambridge Bioscience, U.K.). The annealing reaction contained 10% dimethyl sulphoxide (DMSO), > 50 pmol of sequencing primer (Fig 1), 5 μl template DNA, 40 mM Tris (pH 7·5), 20 mM $MgCl_2$ and 50 mM NaCl in a total volume of 10 μl. The reaction mixture was boiled for 3 min, snap frozen at −70°C. To this was added 0·5 μM each of dCTP, dTTP and dGTP, 1 μl α^{35}S dATP (111 TBq/mmol), 0·025 M DTT and 2 U Sequenase in a total volume of 4 μl. The termination reactions (50°C for 3 min) were performed using the manufacturer's mixes with the addition of 10% DMSO and the products resolved in a denaturing 6% polyacrylamide sequencing gel.

Clone specific probes. Probes, 20 nucleotides in length, were selected from the 'clone specific' V–N–D junctions as demonstrated by sequence analysis, synthesized commercially and purified by gel filtration (Oswell DNA Services, Edinburgh). These were end labelled by a standard T4 polynucleotide kinase technique with γ^{32}P dATP (222 TBq mmol) (Sam-

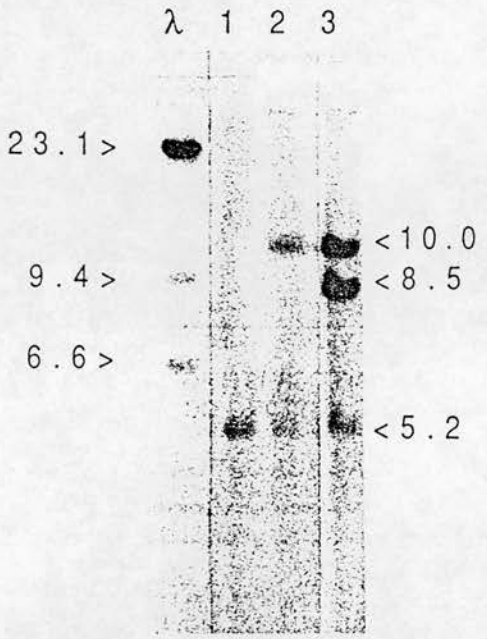

Fig 2. Southern blot analysis of BglII digested DNA hybridized with the JδS16 probe. Normal peripheral blood DNA served as a control (lane 1). Lane 2 shows a patient with a deletion of Jδ1 on one chromosome and a Vδ2–Dδ3 rearrangement on the other. Lane 3 shows a patient with a biallelic rearrangement. Vδ2–Dδ3 and Dδ2–Dδ3. HindIII digested bacteriophage lambda DNA was used as a molecular weight marker.

brook *et al.* 1980). 2 μl of alkali denatured PCR reaction product were slot blotted onto nylon membranes, prehybridized at 65°C for 1 h and hybridized at 65°C for 1 h with the appropriate clone specific probe. Filters were washed (2 × 10 min, RT) with 2 × SSC followed by a 30 min wash at 55°C with 0·1 × SSC containing 0·1% SDS. Filters were exposed to X-ray film for 2 h.

RESULTS

Southern blotting

In 22 patients (10 adults, 12 children) sufficient material was available from presentation marrow to permit Southern blot analysis of TCRδ gene rearrangements using the $J\delta_1$ probe. Rearrangements or deletions were found in 15 patients (68%). In two patients, both alleles were rearranged and in four cases one allele was deleted. Vδ2–Dδ3 rearrangements were detected in 12 patients (55%) and Dδ2–Dδ3 rearrangements in five patients including two with a biallelic rearrangement involving Vδ2–Dδ3 (Fig 2).

PCR and direct sequence analysis

For PCR analysis, presentation samples from all 36 patients were amplified using the Vδ2 and Dδ3 primers (Fig 1). The products obtained from the PCR reaction were analysed by 4% agarose gel electrophoresis (Fig 3). The size of the product varied between 340 and 380 bp indicating a range in size at

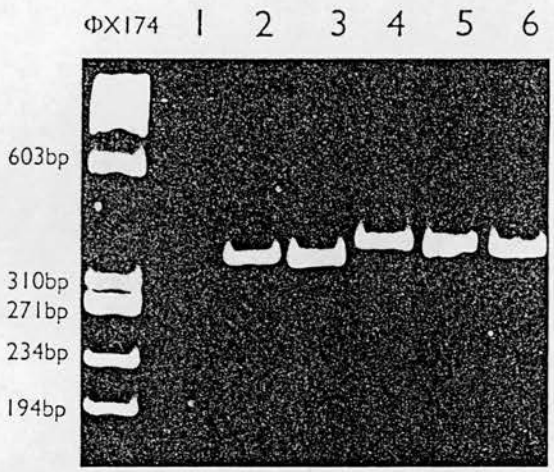

Fig 3. Polyacrylamide gel electrophoresis (5%) of PCR products from five patients (lanes 2–6) and a pooled normal peripheral blood control (lane 1) visualized by ethidium bromide staining. HaeIII digested ϕX174 DNA served as a molecular weight marker. Band size of the PCR product varied from 340 to 380 bp.

the junctional Vδ2–Dδ3 region of approximately 40 bp. PCR analysis demonstrated 19 patients (53%) with a Vδ2–Dδ3 gene rearrangement. All those patients who had a Vδ2–Dδ3 rearrangement as detected by Southern blotting were positive when analysed by PCR and in addition seven of 14 patients not analysed by Southern blotting were PCR positive. PCR products from 18 patients (seven adults, 11 children) were sequenced directly using an internal sequencing primer (Fig 1) to reveal the Vδ2(D)Dδ3 junctional rearrangement. Table I shows the junctional sequences aligned with the published germline configuration for Vδ2, Dδ1, Dδ2 and Dδ3 (Loh *et al.* 1989; Takihara *et al.* 1989). An extensive degree of variation can be seen in the junctional region brought about by deletion of Vδ2 and Dδ3 sequences, inclusion of Dδ2 sequences, P nucleotides and varying amounts of N-nucleotide insertion. The size of the junctional regions ranged from 0 up to 13 bp with an average of 7 bp. Junctional sequences were considered to be derived from Dδ1 or Dδ2 genes if at least three consecutive nucleotides could be aligned with germline sequences. There was no evidence for the presence of Dδ1 derived nucleotides whereas Dδ2 sequences were present in six junctions. Regions of randomly inserted N-nucleotides were seen in 15 patients with an average length of 5·5 nucleotides. In addition, these insertions were found to be GC rich (79% GC content). The amount of nucleotide deletion in the germline sequences of Vδ2 and Dδ3 varied from none to complete deletion of the Dδ3 gene in three patients. The average deletion of nucleotides from Vδ2 was 3·7 (range 0–15) and from Dδ3 and adjacent sequences was 6·0 (range 0–24). Intact gene segments were present in four patients with both intact Vδ2 and Dδ3 present in one patient. P-region nucleotides, defined as forming a palindromic sequence with adjacent nucleotides of an untrimmed gene segment, were present in five patients.

Table I. Sequence analysis of TCR Vδ2–Dδ3 junctions amplified from presentation bone marrow in 11 children and seven adults with cALL. Germline sequences of Vδ2, Dδ1, Dδ2 and Dδ3 are shown. The presence of Dδ2 sequences in the junction is denoted by broken underlining and P-nucleotides by bold italics. Other sequences in the junction are N-nucleotides.

	Vδ2	Dδ1	Dδ2	Dδ3	
GERMLINE	CTGTGCCTGTGACACC	GAAATAGT	CCTTCCTAC	ACTGGGGGATACG	CACAGTGCTACAAA
CHILD cALL					
RK	CTGTGCCTGTGAC	GTGTCTCCGTC		GGGATACG	CACAGTGCTACAAA
EC	CTGTGCCTGTGACAC				AAA
CA	CTGTGCCTGTGAC	CGA		GGGATACG	CACAGTGCTACAAA
CW	CTGTGCCTGTGACACC	***GG***GGGG		TGGGGGATACG	CACAGTGCTACAAA
HM	CTGTGCCTGTGACAC	GGGG		CTGGGGGATACG	CACAGTGCTACAAA
MC	CTGTGCCTGTGACAC	***GG***CCTT		TACG	CACAGTGCTACAAA
JM	CTGTGCCTGTGA	GACCCAC		GGGGATACG	CACAGTGCTACAAA
SM	CTGTGCCTGTGACAC	TCCCCTGGGGG		TGGGGGATACG	CACAGTGCTACAAA
LR	CTGTGCCTGTG	CCCCCCGGA			ACAGTGCTACAAA
MK	CTGTGCCTGTG	GTCT***GT***		ACTGGGGGATACG	CACAGTGCTACAAA
KM	CTGTGCC	CCCC		GGGGGATACG	CACAGTGCTACAAA
ADULT cALL					
WL	CTGTGCCTGTG	CCACAG		GGGGGATACG	CACAGTGCTACAAA
DP	CTGTGCCTGTGACAC	GCA		GGATACG	CACAGTGCTACAAA
GG	CTGTGCCTGTGAC	CCGACCCCCATGG		TGGGGGATACG	CACAGTGCTACAAA
BM	C	CGTCGCCGG			GTGCTACAAA
DT	CTGTGCCTGTGACAC	TCCCCATT***GT***		ACTGGGGGATACG	CACAGTGCTACAAA
TM	CTGTGCCT	CCCTTCGGAC		CG	CACAGTGCTACAAA
HS	CTGTGCCTGTGACACC	CCT***T***		ACTGGGGGATACG	CACAGTGCTACAAA

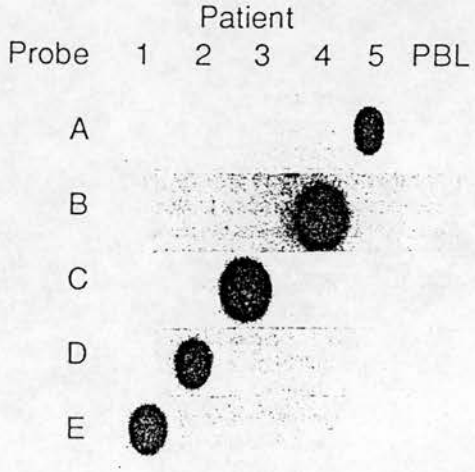

Fig 4. Hybridization of clone specific probes from five patients to amplified DNA samples from presentation bone marrow and of pooled normal peripheral blood control (PBL). Replicate slot blots were hybridized with each individual clone specific probe with probe A being derived from patient 5, etc.

Clone specific probes

From the sequence data of five patients (DP, WL, BM, DT and GG), 20 bp oligonucleotide probes from across the junctional region were synthesized by standard methods. These were radio labelled and used to test both the specificity and sensitivity characteristics of the probes. The specificity was determined by amplifying presentation samples from these patients and from a pool of normal peripheral blood lymphocytes obtained from healthy individuals. Each of the clone specific probes was hybridized to five other cALL patients and to the normal pool. The results are shown in Fig 3. None of the clone specific probes hybridized to the normal pooled DNA or to any other individual cALL patient who had demonstrated a clonal rearrangement of TCRδ. Therefore 20 bp probes are of sufficient length to be specific for their derived clone. The sensitivity of the clone specific probes was determined by mixing experiments. DNA from patients was mixed in serial 10-fold dilutions with DNA from pooled normal lymphocytes. PCR amplified and hybridized with the patient specific probe. The resolution ability of the clone specific probe was approximately one tumour cell in 10^4 normal cells (Fig 4). Clone specific probes from 10 patients were used to analyse remission blood and marrow samples

Table II. Clinical and PCR status during treatment in 10 patients with cALL. Clone specific probes for analysing remission samples were derived from sequenced PCR products from presentation bone marrow (pres BM) or marrow obtained at relapse (rel BM). Clinical remission was determined by morphologic and immunophenotypic criteria.

Patient	Probe source	Clinical status	PCR status
WL	Pres BM	Remission/+1 month died from other causes	+ve during remission
DP	Pres BM	Remission/+23 months relapsed	+ve during remission/PBSCH +ve BMH +ve
DT	Pres BM	Remission/+2 months relapsed/+6 months PBSCT/ remission+7 months relapsed/+8 months died	+ve during remission/PBSCH −ve −ve following PBSCT
BM	Pres BM	Remission not achieved/+3 months died	+21 d BM and B −ve/+28 d −ve
GG	Pres BM	Remission/+4 months BMT/+14 months relapsed/ remission/+33 months relapsed/remission/ +40 months relapsed/+43 months died	+ve during remission/BMH −ve +ve following BMT
RK	Pres BM	Remains in remission at +24 months	+ve up to +5 months/remains −ve at +24 months
CW	Rel BM	Remains in remission at +23 months	+14 d BM −ve/remains −ve at 23 months
MC	Pres BM	Remains in remission at +23 months	+ve up to +16 months/remains −ve at +23 months
CA	Rel BM	Remission not achieved/+10 months, matched unrelated donor transplant/remission/ +18 months relapsed	+ve during remission following BMT
EC	Rel BM	Remission not achieved/+1 month died	Remained +ve

for up to 2 years following induction treatment at diagnosis or relapse (Table II and Fig 5). In three of eight patients who attained remission, the PCR status became negative at varying times following treatment (CW, 14 d; RK, 5 months; MC 16 months). At 23 months all three patients were in complete remission and remained consistently PCR negative. The majority of patients analysed were PCR positive during periods of remission which lasted from 2 to 23 months. Intensive therapy including bone marrow transplant, peripheral blood stem cell (PBSC) transplant or matched unrelated donor transplant had no effect on PCR status. In addition, tumour was detected in bone marrow harvested during remission and PBSC harvests collected in recovery phase.

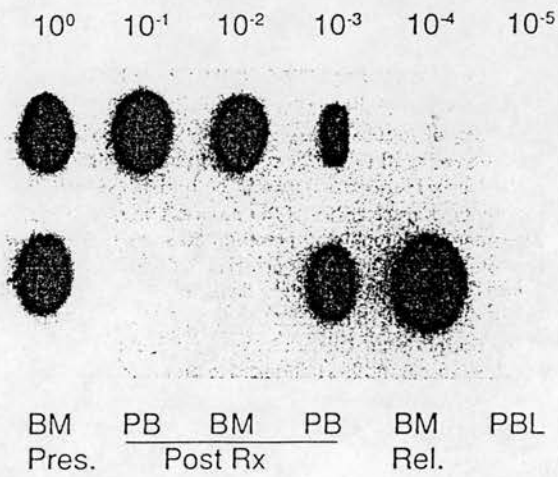

Fig 5. Slot blot analysis of serial dilutions of tumour DNA in normal PBL DNA and of samples collected throughout therapy from patient BM. Bone marrow (BM) and peripheral blood (PB) samples taken following ALL type chemotherapy showed no evidence of tumour. However, a second peripheral blood sample taken within 1 week of the first blood and marrow samples demonstrated a clonal Vδ2–Dδ3 rearrangement. The patient went on to clinically relapse as was evident by a bone marrow sample (BM Rel) taken 6 weeks later.

DISCUSSION

To investigate TCRδ gene rearrangements as a potential tumour marker which may be analysed by PCR amplification, we screened a group of 22 cALL patients by Southern blot analysis. A Vδ2–Dδ3 recombination was present in the majority (12/15, 80%) of our patients with a TCRδ rearrangement. Yokota *et al* (1991b) studied 201 children as part of a large multicentre trial in Germany and found TCRδ rearrangements in 162 (81%) of which Vδ2–Dδ3 was the most frequent recombination in 57% of these patients. In a recent study on 32 children with cALL, Vδ2–Dδ3 was also the most frequent recombination occurring in 78% of 18 patients with a rearrangement at the TCRδ locus (Yano *et al.* 1991). The overall incidence of Vδ2–Dδ3 rearrangements in our group of patients was 55%. In the two other studies on consecutively sampled patients the reported overall incidence of Vδ2–Dδ3 recombination was 92/201 (46%) (Yokota *et al.* 1991b) and 14/18 (44%) (Yano *et al.* 1991). From these results on a combined total of 255 patients, Vδ2–Dδ3 rearrangements are a useful clonal marker, present in approximately one half of patients with cALL.

On the basis of these results we next investigated the frequency of Vδ2–Dδ3 rearrangements in 36 patients by PCR analysis. This confirmed the Southern blot analysis with 55% of patients having a clonal PCR marker. We reasoned to directly sequence the PCR products from the putative Vδ2(D)Dδ3 recombination to provide the following: (i) further characterize this rearrangement including D segment involvement, (ii) to confirm the diversity of the junction which varied in size by only 40 bp, (iii) to construct clone

specific probes from the sequence data for minimal residual disease studies and thus remove the need for nested PCR techniques as described in other studies (Yokota *et al.* 1991b). Our sequence data confirms the extensive diversity of the incomplete TCR Vδ2–Dδ3 rearrangement. This is generated by several factors including insertion of Dδ2 gene segments and N-region nucleotides, the presence of P-region nucleotides and variable deletions of the 3′ end of Vδ2 and 5′ end of Dδ3 genes. Dδ2 gene derived nucleotides were present in 33% of our sequenced patients with Dδ1 sequences absent. Overall these nucleotides contributed 18% of the junctional region. N-nucleotides contributed substantially to the junction, forming on average 75% of nucleotides involved. They were predominantly GC rich (79%) due to the action of the enzyme, terminal deoxynucleotidyltransferase (TdT) which adds template independent nucleotides to the terminals of combining gene segments and has a substrate preference for GC or CG base pairs (Desiderio *et al.* 1984). A third type of junctional feature, termed P-nucleotides, was first described by Lafaille *et al* (1989) as a model for V–D–J recombination based on the frequent observations of mononucleotides or dinucleotides in palindromic sequence with intact joining gene segments and are therefore germline encoded. These were associated with five of nine intact Vδ2, Dδ2 or Dδ3 genes. Exonuclease activity results in 'nibbling' or deletion of the terminals of the recombining gene segments. This was a major feature of the rearrangements which were sequenced. In some patients deletion was extensive, involving 15 nucleotides from Vδ2 in one patient and in three patients the complete Dδ3 gene segment. In only three and two cases respectively were the Dδ3 and Vδ2 gene segments intact.

In a recent study Yokota *et al* (1991a) published sequence data on Vδ2–Dδ3 rearrangements in nine children with cALL. When compared to our sequence data several differences are evident. Firstly, Yokota *et al* (1991a) found no convincing evidence for the presence of Dδ1 or Dδ2 gene segments in their junctions, whereas Dδ2 was present in one third of our patients. Secondly, they reported an extremely high incidence of intact 5′ ends of Dδ3 (8/9, 89%) and although this is not a characteristic feature of cALL, they suggested it may preferentially occur in this type of leukaemia. However, we report only three of 18 patients (17%) with intact 5′ Dδ3 ends. In addition there was a higher frequency of P-nucleotides associated with the intact Dδ3 ends (67% *v* 29%). Using direct sequence analysis of PCR products from a normal lymphoid population, the incidence of intact Dδ3 ends was reported as 50% (Loh *et al.* 1989; Takihara *et al.* 1989; Yokota *et al.* 1991a). There was no obvious explanation for the low incidence of intact 5′ Dδ3 boundaries in our patient group. Patients were selected consecutively at presentation and classified by standard phenotypic methods. However, the varied deletion of the Dδ3 end contributed to the extensive diversity in the Vδ2–Dδ3 junction which we report in our patient group.

From the sequence data we designed clone specific probes of 20 nucleotides in length using, where possible, the variation encoded by inserted sequences and deletions at the Vδ2–Dδ3 junction. We have referred to these as clone specific probes to differentiate from the term clonospecific probes which are generated directly from nested PCR amplification of the Vδ2–Dδ3 junction and which are approximately 300 bp in length (Yokota *et al.* 1991b). In hybridization experiments, clone specific probes did not cross-react with amplified tumour from other patients or with normal pooled bone marrow. The sensitivity of the clone specific probes directed against the partial Vδ2–Dδ3 rearrangement was 10^{-4}. This reduced sensitivity was predicted (van Dongen *et al.* 1992) with 10^{-6} being the theoretical detection limit for PCR amplification using 10 μg template DNA. The detection limit is in part determined by the size of the junctional region, which in our patients averaged 6·83 nucleotides. In complete Vδ1–Jδ1 rearrangements, with junctional inserts up to 37 nucleotides in length, highly specific clone specific probes can be designed capable of detecting leukaemic cells down to a frequency of 1 in 10^6 (van Dongen *et al.* 1992). The presence of other non-random sequences in the Vδ2–Dδ3 recombination including Dδ2 and P-nucleotides and the GC rich nature of the randomly inserted N-nucleotides may contribute to a reduction in the potential detection limits of these probes for minimal residual disease analysis. Clone specific probes derived from Vδ2–Dδ3 were able to detect residual tumour in blood and marrow obtained during clinical remission. Serial analyses of remission samples showed residual tumour may either persist for periods of up to 2 years before the onset of relapse or may become undetectable by PCR indicating possible eradication of tumour following therapy. In the latter, loss of PCR detectable disease occurred at varying times after treatment and these patients continued to be in remission at 2 years. These two patterns, loss of detectable tumour and persistence of disease, were reported in ALL by other groups using different PCR clonal markers (IgH CDRIII, Yamada *et al.* 1990; TCR Vδ–Jδ, Neale *et al.* 1991; TCR Vδ2–Dδ3, Yokota *et al.* 1991b). Based on these reports with similar sensitivities, there was considerable variation in the persistence of tumour during remission. From our results there was loss of tumour in one patient within 14 d of treatment whereas other groups have reported the persistence of tumour in remission at 3 years (Campana *et al.* 1990b). This may indicate that complete elimination of residual disease is not an essential prerequisite for long-term survival, although in our group the continued presence of tumour up to 2 years resulted in relapse. The small numbers studied in these reports and the variation in tumour response makes it difficult to interpret PCR analysis of remission samples as having a predictive value for long-term survival. However, Neale *et al* (1991) have suggested those patients who are PCR negative within 16 months of diagnosis may have a better prognosis. The continued presence of residual tumour may have an effect on stem cell transplantation where bone marrow and PBSC are harvested during remission. Both PBSC and bone marrow harvests were contaminated with tumour and although high-dose therapy and stem cell transplantation induced remission it did not result in eradication of PCR detectable tumour. Whether this indicated chemoresistant tumour or the inability of high-dose therapy to reduce tumour levels below the threshold of PCR analysis was not clear. Double-colour immunofluorescence is capable of detecting cells expressing leukaemic phenotypes at an

equivalent sensitivity to that of PCR based techniques (Campana *et al.* 1990a). Although standard antibody reagents are used in a simpler experimental technique and more applicable to routine analysis, immunological analysis of minimal residual disease is limited to 35% of pre-B ALL patients and can also generate false negative results in 20% of patients who relapse (Bregni *et al.* 1989). Combined immunological and PCR analysis was recommended (Campana *et al.* 1990b) and features such as clonal instability (Potter *et al.* 1992) and oligoclonality (Tycko *et al.* 1992) may be effectively monitored using PCR analysis based on two or more different techniques (TCRδ, TCRγ or IgH CDRIII).

Our technique for generating clone specific probes to Vδ2–Dδ3 gene rearrangements differs from that described by Yokota *et al* (1991b) who used the amplified junction product as a clonospecific probe. We elected to directly sequence the Vδ2–Dδ3 junction from amplified presentation sample and synthesize 20b oligonucleotide using the sequence data. This allowed for a standard stringent wash to be used following hybridization, whereas PCR derived clonospecific probes required a high stringent final wash (0·05 × SCC at 68°C). This was presumably because these probes contained sequences from minor populations of normal cells present in the amplified tumour sample. Synthetic oligonucleotide probes derived from sequence data in T-ALL (Neale *et al.* 1991) were also reported to have a greater specificity in detecting leukaemic clones than techniques based on using probes derived from the PCR product (Hansen-Hagge *et al.* 1989).

Although PCR techniques to monitor minimal residual disease are becoming more easily adapted for routine laboratory analysis, there remain several limitations to the technique. Firstly changes in clonality or subclonal formation during treatment may lead to false-negative results. Changes in TCR gene rearrangements have not been widely reported; however, this problem is associated with IgH gene analysis where subclonal formation is reported to occur in 15–30% of precursor B-ALL (van Dongen & Wolvers-Tettero, 1991). Secondly, the restricted size of the junctional region in the incomplete TCRδ gene rearrangement influences the sensitivity of the clone specific probes making it more difficult to quantify tumour contamination in remission samples. Lastly, the clinical significance of extremely low levels of tumour cells during remission has not been completely evaluated. At present, PCR data on longitudinal studies of patients with ALL during treatment is limited; however, the ability to study greater numbers of patients using TCR Vδ2–Dδ3 as a clonal marker will make these long-term objectives more feasible.

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Clonal Selection in Acute Lymphoblastic Leukaemia Demonstrated by Polymerase Chain Reaction Analysis of Immunoglobulin Heavy Chain and T-Cell Receptor δ Chain Rearrangements

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Immunoglobulin heavy chain (IgH) and T-cell receptor (TcR) genes can be monitored as markers of clonality by polymerase chain reaction (PCR) analysis in acute lymphoblastic leukaemia (ALL). We report the short clinical course of a 16-year-old patient with ALL and a t(4;11) who relapsed early following treatment and subsequently received reinduction chemotherapy followed by peripheral blood stem cell transplantation with interleukin 2 therapy. Despite this, the patient relapsed and died 8 months after presentation. The leukaemic cells were analysed by PCR and showed rearrangements of TcR Vδ2-Dδ3 and IgH CDRIII genes. Direct sequence analysis of the TcRδ and IgH PCR products revealed two leukaemic clones at diagnosis with one present at minimal levels. After initial therapy the major clone was no longer detected even in subsequent relapse samples but the originally minimal clone persisted and increased despite further treatment, indicating drug resistance.

INTRODUCTION

Relapse of disease in patients with haematological malignancies following chemotherapy or stem cell transplantation may result from neoplastic cells persisting throughout treatment or from reinfusion of tumour contaminating stored stem cells. This has previously been detected by cytogenetics, immunophenotyping, and Southern blotting with immunoglobulin heavy chain (IgH) and T-cell receptor (TcR) probes. These approaches can detect clonal populations of cells only when they exceed approximately 2–5% of the sample under study (1) .The advent of polymerase chain reaction (PCR) analysis has enabled the investigation of residual disease at a sensitivity of one cell in a background of 10^5 normal cells (2). Both IgH and TcRδ genes can be amplified in a proportion of patients with acute lymphoblastic leukemia (ALL). Using primers complementary to TcR Vδ2 and Dδ3 genes, the intermediate junctional regions can be amplified, and used as clone-specific probes (3). Similarly, using primers homologous to consensus V_H and J_H gene segments, oligonucleotide probes to the intervening third complementarity-determining region (CDRIII) of IgH can be designed (4).

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LEUKEMIA

PCR-based antigen receptor gene analysis may have limited application in patients who present with multiple gene rearrangements (oligoclonality) or who acquire new rearrangements at relapse (clonal evolution). This risks a false negative assessment of residual disease. The IgH rearrangement may be a particularly unstable tumour marker. Forty-five percent of patients with B-cell ALL were found to have an oligoclonal or biclonal IgH rearrangement at presentation (5) and clonal evolution was a frequent event in 50% of these patients (6). In a recent case report of childhood ALL, a complete clonal change was evident during the initial treatment period within 10 months of presentation (7). TcRδ rearrangements are thought to be a more stable marker, although the presence of biallelic TcR Vδ2-Dδ3 rearrangements (8) may make it more difficult to design tumour specific probes.

We present a case of a patient with a t(4;11) ALL in whom IgH and TcR rearrangements were detectable by PCR. The TcRδ rearrangements were exploited to generate tumour-specific probes to two clones present at diagnosis. Monitoring of sequential samples taken throughout the patient's clinical course demonstrated that one clone was chemoresistant and led to relapse. Despite reinfusion of the chemosensitive clone at the time of peripheral blood stem cell (PBSC) transplantation it could not be detected in any further marrow samples up to the patient's death.

CASE REPORT

A 16-year-old male presented with symptoms of anaemia, hepatosplenomegaly and fundal haemorrhages. Peripheral blood showed haemoglobin 63 g/l, the white cell count was $151 \times 10^9/1$ with 98% blast cells, and platelets at $42 \times 10^9/1$. Bone marrow aspirate confirmed early pre-B-cell ALL. The blasts demonstrated terminal deoxytransferase (TdT), class II and CD19 positivity, but were negative for CD10, CD20, and surface immunoglobulin. Cytogenetic analysis revealed a t(4;11) translocation.

Induction treatment was according to the UKALL XA schedule. The day 21 bone marrow aspirate showed morphological remission and no evidence of ALL by immunophenotyping or cytogenetic analysis. Peripheral blood stem cell harvesting (PBSCH) was performed using a Cobe Spectra as his white count was recovering and the cells were cryopreserved. After UKALL XA intensification chemotherapy, remission appeared to be

maintained. Further PBSCH were cryopreserved. Cranial irradiation and maintenance therapy were commenced.

Two months later the patient experienced general malaise, and bone marrow relapse was confirmed by morphology, immunophenotyping, and cytogenetic studies. Morphological and immunophenotypic remission was achieved with mitozantrone 20 mg/m^2 for 2 days and cytosine arabinoside 3 g/m^2 for 5 days, with further PBSCH obtained on recovery. Cytogenetic studies however showed the presence of a t(4;11) in 10% of the cells, one-third of which also had 7q− and monosomy 18.

As no HLA identical sibling was available, peripheral blood stem cell transplantation (PBSCT) with interleukin 2 (IL-2) to increase graft versus leukaemia was undertaken after cyclophosphamide and total body irradiation conditioning. Morphological, immunophenotypic, and cytogenetic remission was achieved. One subsequent course of IL-2 was administered but 7 weeks after PBSCT, the patient relapsed morphologically and died only 8 months after presentation.

MATERIALS AND METHODS

DNA Extraction

Peripheral blood (PB) and bone marrow (BM) mononuclear cells were separated over Ficoll-Hypaque (density 1.077 g/l, Pharmacia, Uppsala, Sweden), lysed, digested with proteinase K and high molecular weight DNA extracted with phenol/chloroform (9).

PCR Amplification

TcR Vδ2-Dδ3 junctions were amplified using primers detailed in Table 1 (3). For the IgH CDRIII junction, consensus primers for the variable (V) and joining (J) domains were used (Table 1)(10). The 19 base pair (bp) J amplimer was homologous to a core region shared by all six J region segments. PCR amplification was performed under standard conditions (2) with 1 U Taq polymerase (Applied Biotechnologies, London, UK) per 100 μl reaction and either 2 mM (TcR) or 1 mM $MgCl_2$ (CDRIII). Amplification consisted of 94°C for 90 s , 56°C for 60 s and 72°C for 90 s for 35 cycles with an extension step of 7 min in the final cycle. One microgram of post-therapy DNA or 0.1 μg of presentation DNA was amplified in duplicate. Products were analysed by 6% polyacrylamide gel electrophoresis (PAGE) stained with ethidium bromide.

Table 1. Sequences of Primers.

Locus	Primer	pmol
IgH		
PCR and sequencing primers (10)		
V_H	5' - ACACGGC$^{C}\backslash_{T}{}^{G}\backslash_{C}$TGTATTACTGT - 3'	25
J_H	5' - ACCTGAGGAGACGGTGACC - 3'	25
TcR Vδ2-Dδ3		
PCR primers (3)		
Vδ2	5' - GAGTCATGTCAGCCATTGAG - 3'	30
Dδ3	5' - AGGGAAATGGCACTTTTGCC - 3'	30
Sequencing primer		
Vδ2	5' - GCACCATCAGAGAGAGATGA - 3'	50

Direct Sequence Analysis

TcRδ and CDRIII PCR products were resolved in 4% NuSieve GTG agarose (FMC Bioproducts, Denmark) and the relevant band purified using glass beads (Geneclean, Stratech Scientific, UK). A modified (11) dideoxy-termination sequencing method (12) was used on the double-stranded PCR products.

Detection of Leukaemia

From the sequence data, specific anti-junctional oligonucleotides were selected against the junction of the TcR Vδ2-Dδ3 rearrangement and obtained commercially (Oswell DNA services, Edinburgh, UK). DNA samples to be probed were amplified as described above, 2 μl of PCR product was alkaline-denatured (13), slot-blotted onto nylon membranes (GeneScreen Plus, Du Pont, UK) and prehybridised in 7% sodium dodecyl sulphate (SDS), 0.5 M NaH_2PO_4 pH 7.5, and 1% bovine serum albumin at 62°C for 1 h. Oligonucleotide probes were end labelled with ^{32}P-dATP (6000 Ci/mmol) using T4 polynucleotide kinase and 1.0×10^6 cpm/ml hybridisation fluid added. After a further 1 h incubation at 62°C membranes were rinsed in 2 × standard sodium citrate (SSC) and washed at 55°C in 0.1 × SSC with 0.1% SDS. Autoradiographs were exposed at −70°C for 2–4 h.

RESULTS

PCR and Direct Sequence Analysis

Amplification of presentation BM demonstrated an IgH band of approximately 110 bp and a TcRδ band of

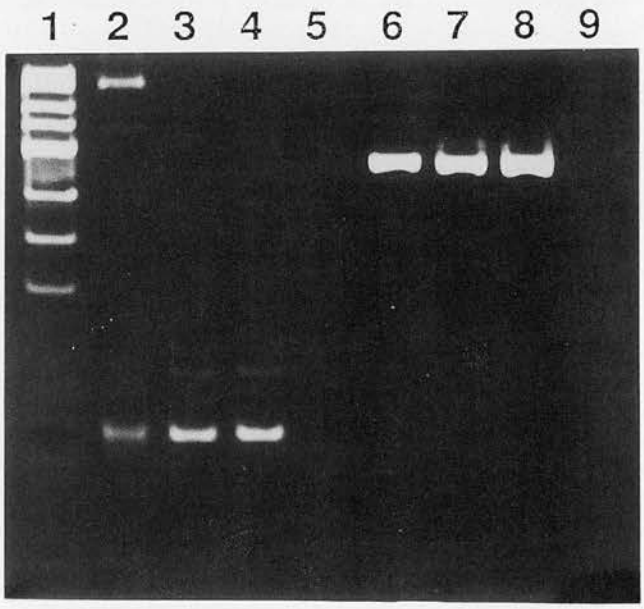

Figure 1. Polyacrylamide gel electrophoresis (6%) of PCR amplification products. Lanes 2–5 contain IgH CDRIII products and lanes 6–9 contain TcR Vδ2-Dδ3 products. DNA from presentation BM is shown in lanes 2 and 6, first and second relapse BM samples are shown in lanes 3 and 7 and in lanes 4 and 8, respectively. Lanes 5 and 9 contain products from pooled normal peripheral blood DNA as negative controls. Lane 1 contains a *Hinf*1 digested φX174 molecular weight marker. A single IgH band at presentation was shown to contain a biclonal IgH rearrangement on sequence analysis which at both relapses was evident as two distinct bands. The size of the relapsed TcR products also differed from presentation.

Table 2. Sequence Analysis of TcR V62-D63 and IgH CDRIII Junctions Amplified from Presentation, First and Second Relapse Bone Marrow Samples.

TcR Vδ2-Dδ3			
	Vδ2	NDN	Dδ3
Presentation			
	CTGTGCCTGTGACAC	TCCCCATT**GT**	ACTGGGGGATACG
1st/2nd Relapse			
	CTGTGCCTG	AGAAGG	GGGGGATACG
IgH CDRIII			
	V_H	NDN	J_H
Presentation			
	CAAGA	GATGTTGAGGGTTATTGTAGTAGTACCAGCTGCTAT (DLR4)	AACTGGTT [J5]
	CGAGA	GAGATCTACGTATTACGATATTTTGACTGGTTAT (DXP4)	AACTGGTT [J5]
1st/2nd Relapse			
	CAAGA	GCATGATATTGTAGTAGTACCAGCTGCCCGGGCCGATATTTTGACTGGTTATTA (DLR4 DXP1)	TACTACTAC [J6]
	CAAGA	GGAGGTGTATGCATTTAT (DLR1)	TACTACTAC [J6]

Inserted D segments are denoted by underlining and P-nucleotides are shown in bold. CDRIII D segments were classified according to Ichihara (21). In TcRδ rearrangements, Dδ2 was involved at presentation and Dδ1 at relapse. A biallelic rearrangement was found for IgH CDRIII genes at presentation and relapse.

350 bp (Figure 1). Analysis of BM DNA at first relapse revealed two IgH bands of approximately 110 bp and 140 bp,along with a TcRδ band of 340 bp. Band sizes at second relapse were identical to those at first relapse. No discrete PCR products were visible in normal control DNA.

TcRδ and IgH PCR products from presentation and relapse BM were sequenced and the results summarised in Table 2. Two distinct clones were identified from TcRδ sequences. Sequences derived from Dδ2 and P-nucleotides associated with an intact Dδ3 gene were present in clone A, whereas in clone B there was deletion of Vδ2 and Dδ3 gene segments, and Dδ1 sequences were inserted. Two distinct biallelic IgH rearrangements were seen by sequence analysis of presentation and relapse BM. The rearrangements involved different J segments (presentation J5, relapse J6), different D segments, and variable N-nucleotide insertion.

Detection of Leukaemia

Clone-specific probes (probe A and B) were designed to the Vδ2-Dδ3 junction from the sequence of clones A and B and used to examine amplified DNA from serial samples collected through the patient's treatment (Figure 2). Both probes could detect tumour at a level of one leukaemic cell in 10^4 to 10^5 normal cells. When hybridised to PB and BM at presentation and the day 21 BM, considered to be in morphological and immunophenotypic remission, probe A demonstrated the presence of tumour (Figure 2a). The PBSCH performed after induction and intensification were also positive (S1, S2). However, the BM at relapse and all subsequent samples were negative. Probe B was hybridised against the same samples (Figure 2b). The presentation samples, day 21 BM and PBSCH following induction and intensification were faintly positive. PBSCH obtained after induction, intensification, and reinduction therapy had minimal contamination with clone B tumour. The relapse BM and post IL-2 treatment BM were all PCR positive for clone B.

DISCUSSION

This case illustrates the typical short clinical course of a young man with a t(4:11) ALL. This leukaemia typically presents with high risk features and although remissions are achieved, these are usually of short duration (14–16). Despite conventional and experimental treatment including PBSCT and IL-2 therapy, our patient relapsed early and died from resistant disease. Although entering remission on morphological, immunophenotypic, and cytogenetic analyses, it was apparent on molecular studies that the leukaemia exhibited two clones at presentation, one of which was sensitive to chemotherapy. The second clone, which although present in low amounts at diagnosis, became predominant at relapse and persisted despite reinduction chemotherapy, PBSCT, and IL-2 therapy.

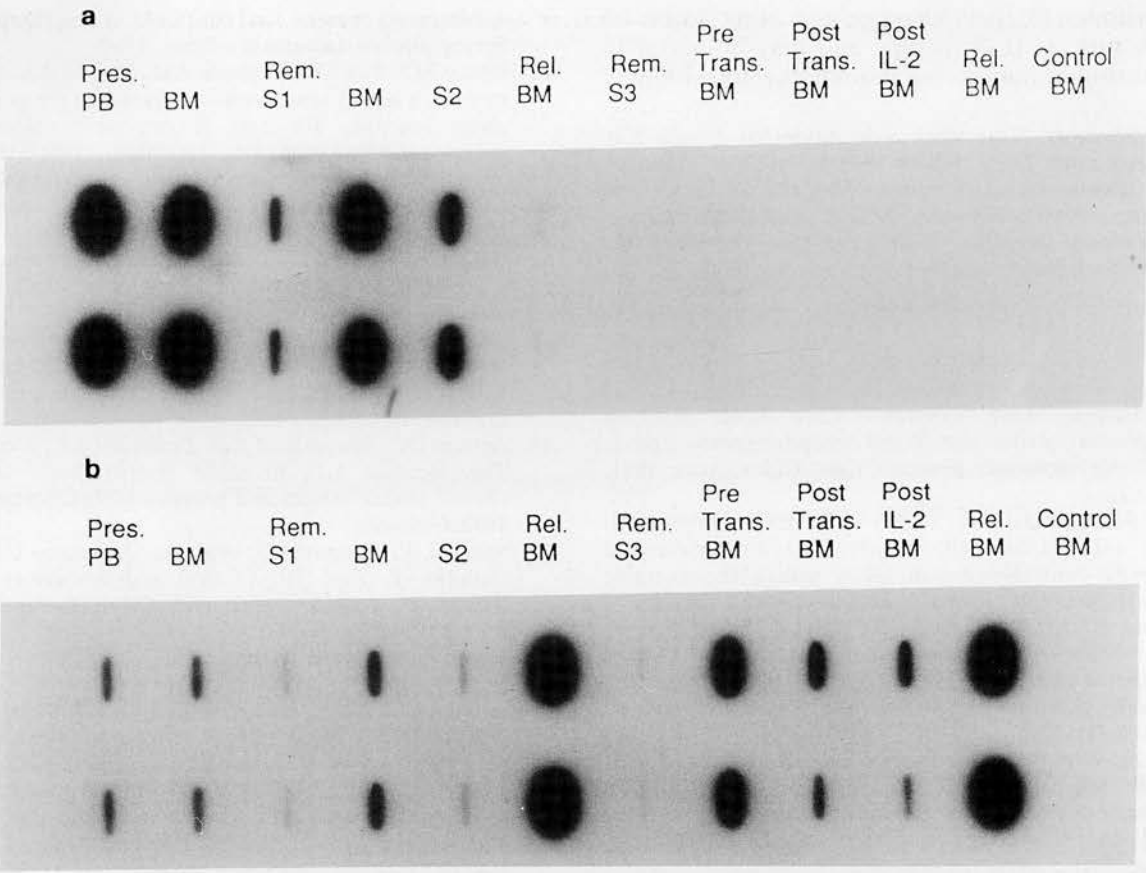

Figure 2. Autoradiograph of replicate slot blots of patient's sequential TcRδ amplification products hybridised with clone-specific probes A and B. Clone A was present in presentation (Pres.) peripheral blood (PB), bone marrow (BM), PBSCH after induction (S1) and intensification (S2), and in the apparent remission bone marrow aspirate taken on day 21 (BM). Clone B was apparent in all of the above samples and additionally in the first relapse (Rel.) bone marrow, PBSCH taken after reinduction (Rem. S3), and bone marrows taken pre- and post-PBSC transplant and IL-2. The final relapsed bone marrow was heavily contaminated when probed for clone B. Negative control consisted of pooled normal bone marrows (Control BM).

The clinical relevance of PCR-detected contamination in reinfused PBSCH or bone marrow is still unknown. Despite receiving a PBSCT with harvests containing clone A, this was not detected at subsequent relapse and therefore, in our patient, this source of tumour was not clinically important.

This case is one of a proportion of ALLs amenable to PCR analysis using two tumour markers. Approximately 80% of patients with B-lineage ALL can be studied with CDRIII primers (4), although the use of family-specific VH primers can increase this percentage (17). Biondi (18) reported a 50% incidence of Vδ2-Dδ3 partial recombinations in patients with common ALL. However this case also highlights some of the problems associated with PCR-based systems using IgH and TcR genes as clonal markers for tumour detection. An important feature of PCR analysis is that the leukaemic cells form the majority clonal population. This can be identified as discrete bands on polyacrylamide gel electrophoresis which, to generate clone-specific probes, are either sequenced (4) or the bands extracted and used as a probe (3). However problems may occur where there is a biclonal or oligoclonal rearrangement with each clone present in differing proportions, as was demonstrated in this case. PAGE alone indicated a monoclonal rearrangement at presentation, which after induction and intensification therapy was replaced by a second neoplastic clone at relapse. Sequence analysis permitted detailed examination of the rearrangements and synthesis of two oligonucleotide probes which on subsequent reprobing of all samples revealed the presence of a biclonal rearrangement at presentation. The complete loss of one clone and persistence of a second during therapy suggests that the second clone was chemoresistant, and selected for by treatment. Clonal evolution or V_H gene replacement, which has been reported in 30% of childhood ALL patients (19), was, therefore, not a feature of this patient's tumour. In a recent case report of early pre-B-cell ALL, a biclonal IgH gene rearrangement was stable during the course of the disease which included an initial remission of 5 years (20). In contrast, a single TcRγ gene rearrangement at diagnosis was replaced completely with an unrelated biclonal gene rearrangement at relapse.

In conclusion, the presence of a biclonal or oligoclonal IgH or TcR rearrangement at presentation reduces the effectiveness of monitoring residual disease by clone-specific probes as they cannot predict which subclone will be responsible for relapse. However, clone-specific probes are useful in monitoring the response of indiv-

idual subclones to chemotherapy and other forms of treatment such as IL-2 therapy and may be useful in combined studies monitoring tumour chemoresistance.

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